PHARMACOGENOMICS OF CHEMOTHERAPEUTIC AGENTS: CARBOPLATIN AND PACLITAXEL

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DEDICATION

To my beloved companion, Amit

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CHAPTER 1 LITERATURE REVIEW

1.1 Ovarian cancer

1.1.1 Introduction

The ovaries are the most important part of the female reproductive system [1]. Each ovary is divided into an outer cortex and an inner medulla [1]. The surface of the ovary is covered by a single layer of cuboidal epithelium, called germinal epithelium[1].

Ovarian cancer is a type of cancer that begins in the ovaries and often goes undetected until it has spread within the pelvis and abdomen [2]. It is ninth most common cause of cancer death among women in the United States. According to the Cancer Facts & Figures published by the American cancer society (ACS), 21, 290 (12.1 per 100,000 women per year) new cases and 14,180 deaths (7.7 per 100,000 women per year) due to ovarian cancer were registered in 2015 in the United States. Five year survival rate was 45.6% (**Figure 1.1**) [3]. Broadly, tumors of the ovaries are classified into three major categories, i) Ovarian epithelial cancer, ii) Ovarian germ cell tumor and iii) Ovarian low malignant potential tumor.

Epithelial ovarian cancer (EOC) is one of the most common gynecological malignancies and the fifth leading cause of cancer death among women in the United States (6% of cancer deaths) [4]. About 9 out of 10 tumors of the ovary diagnosed (90%) are of this type. EOC occur primarily in middle aged or older women while it is rare in young adults, especially before puberty [5]. The standard treatment for patients with advanced disease is initial debulking surgery followed by carboplatin-paclitaxel combination chemotherapy. Five-year overall survival (OS) remains around 44.2%, suggesting there is a wide inter-patient variation in response [6].

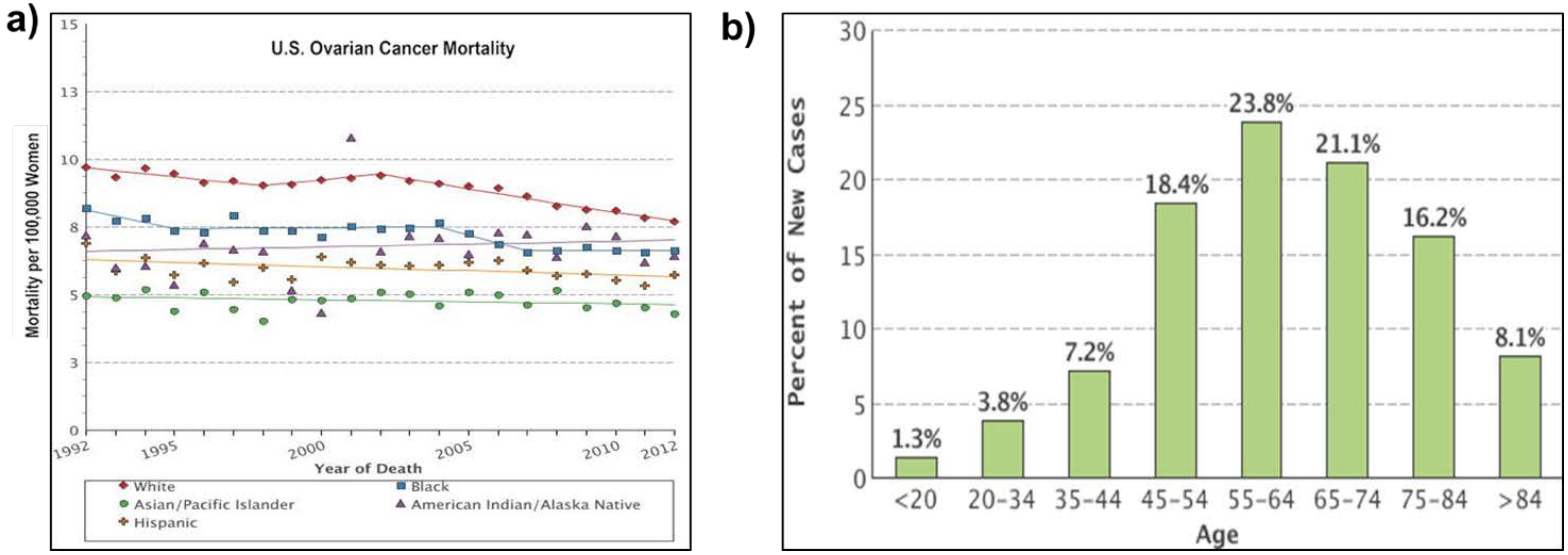


Figure 1.1 a) Incidence of ovarian cancer in the US, 1992-2012. b) New cases, 2008-2012, All Races, Females (NCI SEER cancer statistics)

1.1.2 Risk factors

Factors like genetic mutations, hormonal therapy, age, nulliparity, obesity, and fertility drugs are associated with increased risk of ovarian cancer. Whereas oral contraceptives, multiple pregnancies, breast feeding and tubular ligation are associated with decreased risk of ovarian cancer.

a) Genetic factors: Approximately 20% of ovarian cancer is familial and linked with BRCA1 or BRCA2 gene mutation [7, 8]. Germline deleterious mutations in the BRCA1/BRCA2 genes are associated with 15% to 40% lifetime risk of ovarian cancer [9, 10]. Loss of BRCA1 or BRCA2 protein expression is more common in ovarian cancer [11]. Prophylactic measure for this mutation is bilateral oophorectomy and it is beneficial in certain cases [12]. Other gene like TP53, PTEN/MMAC1, STK11 and mismatch repair genes MLH1, MSH2, MSH6, and PMS2, have been associated with an increased risk of ovarian cancer (NCI).

b) Hormonal therapy: Women using more than 10 years of hormonal therapy including estrogen only, progestagen only, estrogen-progestagen combinations and tibolone are in increased risk of ovarian cancer. The relative risk (RR) of hormone therapy current users verses never users of hormone therapy was 1.20 (95% confidence interval [CI], 1.09–1.32). Risk increases with increasing duration of use [13]. A population-based case-control study of exclusively estrogen-only therapy for at least 5 years showed odds ratio [OR], 1.6; 95% CI .A cohort study showed that use of ERT(estrogen replacement therapy)/ET (estrogen therapy) and ERT/ET followed by EPRT (estrogen –progesterone replacement therapy) more than 20 years are associated with increased risk of ovarian cancer (RR=relative risk 3.2 95%CI, 1.7-5.7) [14].

c) Age and Nulliparity: Risk of developing ovarian cancer increases linearly from age 30 to 50 years of age and continues to increase further with age thereafter [15]. Women over 55 are most commonly diagnosed with ovarian cancer. About half of the women who are

diagnosed with ovarian cancer are 63 years or older [2]. Furthermore, there is increased risk of ovarian cancer in older woman who have never been pregnant [16, 17].

d) Body Mass Index (BMI), Diet and Obesity: BMI (= (the weight in kgs)/ (square of height in meters)) is associated with increased mortality rate from ovarian cancer. A prospective study on more than 900,000 U.S. adults (404,576 men and 495,477 women) showed individuals with BMI ≥ 40 had higher death rates from cancers (52% for men; 62% for women) compared to 'normal' BMI (18.5 to 24.9) [18]. Some studies also support the association between increased risk of cancer with height and diet, along with BMI [19-21].

e) Oral contraceptives: Interestingly, use of Oral contraceptive is associated with reduced risk of ovarian cancer. 10%-20% reduction in ovarian cancer risk is associated with one year use of oral contraceptive, where as 50% reduce risk associated with five years use of oral contraceptive among women [22, 23]. Case-control (24) and cohort studies published since 2000 observed risk reduction with use of oral contraceptive more than 1 year [24]. A multicenter study with 330 cases and 982 matched controls, showed a statistically significant decreased risk of ovarian cancer associated with Progestational contraceptives (depot-medroxyprogesterone acetate [DMPA]) use [25].

f) Tubular ligation: Decreased risk of ovarian cancer associated with tubal ligation [26, 27]. Population-based case-control studies (13) showed tubal ligation is associated with a 29% reduction in ovarian cancer risk (OR, 0.71; 95% CI, 0.66–0.77) [28].

g) Breast feeding: Case control studies (30) showed breast-feeding is associated with a decreased risk of ovarian cancer (RR, 0.76; 95% CI, 0.69–0.83) [29].

h) Smoking: A meta-analysis from 51 studies with 28,114 ovarian cancer patient found a very small increased risk of ovarian cancer among current smokers compared with nonsmoker (RR, 1.06; 95% CI, 1.01–1.11) [13].

i) Use of Talc: A meta-analysis of 16 studies observed an increased risk with the use of talc (RR, 1.33; 95% CI, 1.16–1.45) [30]. A case-control study that included 8,525 cases and 9,859 controls have proven increased risk of epithelial ovarian cancer associated with genital powder use (OR, 1.24; 95% CI, 1.15–1.33) [31].

1.1.3 Prognosis of Ovarian cancer

Women with low risk cancers defined as stage IA, IB, grade 1 or 2, nonclear-cell histologies do not need further adjuvant therapy. Survival of early-stage disease is significantly higher (20% to 30%) than advanced stage ovarian cancers [32]. Five-year disease-specific survival is 84% for stage I compared to stage II disease, which is 76% [32]. Interestingly younger women are more likely to be diagnosed with a lower-stage along with more well-differentiated tumors and have an improved outcome compared with older women. In addition, patients with a significant component of transitional cell carcinoma have a better prognosis [33-35]. Case control studies suggest that BRCA1 and BRCA2 mutation carriers show better response compared to sporadic EOC patients [36]. Patients with high-risk early-stage epithelial ovarian cancer defined as stage I, grade 3; stage IC, stage II, clear-cell cancers and they require postsurgical adjuvant treatment. Additionally, late diagnosis is a major problem for ovarian cancer. Lack of awareness of patients and physicians about occurrence of nonspecific symptoms like abdominal pain and swelling, gastrointestinal symptoms and pelvic pain is another major concern. The ovarian cancer-associated biomarker CA-125 is more significant at the time of disease progression after 1-2 chemotherapy cycle in stage III and satge IV, instead of time of early stage of diagnosis [37].

1.1.4 Treatment of Ovarian epithelial cancer

There are various therapies available for EOC which are selected based upon stage and tumor types. Platinum based chemotherapy is the standard-of-care for 75% of ovarian cancer patients. The

treatment of EOC is divided into three groups- Early stage, advanced stage and recurrent or persistence (as shown in **Figure 1.2**).

Standard chemotherapy worldwide for epithelial ovarian cancer are platinum agents cisplatin or carboplatin either alone or combination with cyclophosphamide or paclitaxel [38-42]. This regimen was evaluated and established by several clinical trials by GOG (Gynecology Oncology Group), MRC (Medical Research Council) [43-48]. However, approximately 80% ovarian epithelial cancer patients relapse after first line platinum and taxane based chemotherapy. If CA-125 increases after first line treatment is completed, then relapse is usually suspected. [49]. Patients with recurrent disease are therefore subdivided as: i) Platinum - sensitive recurrent and ii) Platinum - refractory/resistance recurrent [49].

Carboplatin was approved by FDA in 1987 for ovarian cancer patient who are recurrent after cisplatin therapy [50]. Currently, the most commonly used regimens for the Platinum-sensitive recurrent subtype are cisplatin or carboplatin + paclitaxel, carboplatin+ Gemcitabine, carboplatin + pegylated lyposomal doxorubicin [51-56]. Other regimens include carboplatin + epirubicin, Cisplatin + doxorubicin + cyclophosphamide, PEGylated liposomal doxorubin + trabectedin [57]. [58].

If recurrence occurs within six months of completion of platinum containing therapy, it is referred to as Platinum-Refractory or Platinum-resistant Recurrence ovarian cancer. Anthracyclines (PLD), taxanes, topotecan, and gemcitabine are used as single agents for these recurrences, which are primarily contributed by platinum-resistant subsets [59-65]. Paclitaxel is also commonly used in front-line induction regimens [64]. Furthermore, adverse effects of platinum-based chemotherapy in ovarian cancer as a single agent or in combinations are myelosuppression, nausea, vomiting, alopecia, and asthenia for this therapy. The most common toxicities are neutropenia, and gastrointestinal (GI) toxicity.

1.1.5 Ovarian cancer clinical trials using Platinum/Paclitaxel chemotherapy

Platinum drugs such as cisplatin plus paclitaxel combination therapy have been widely adopted as standard treatment for advanced ovarian cancer. A number of Phase I/II/ III clinical trials have evaluated the combination of carboplatin/paclitaxel chemotherapy and shown improvements in treatment outcomes in ovarian cancer patients following administration of platinating agents as chemotherapeutic agents and the use of paclitaxel as combination treatment, as summarized in **Table 1.1.**

Evidently, despite these treatment interventions, drug resistance, disease recurrence, and toxicities are major challenges for platinum-based therapy in ovarian cancer. The clinical trials presented here report that there is considerable variation in response to therapy which is a major challenge for platinum based therapy in ovarian cancer patients.

Table 1.1 Details of clinical trials evaluating platinum drugs/paclitaxel combination therapy in ovarian cancer.

Patient details	Drug response/outcome	Drug Toxicity	Pubmed ID
German Arbeitsgemeinschaft Gynäkologische Onkologie (AGO) patients (n=518)	Overall response rate=75%; Complete responses rate= 41%; Partial response=34%.	Hematologic toxicity occurred more frequently than non-hematologic toxicity	9346222
Stage IV ovarian cancer patients (n=34)	Complete response rate=82%; Partial rate=95%; 1-year survival rate=94%	Nausea and vomiting grade 2=30%; Grade 2-3 gastrointestinal toxicity=20%; Grade 3-4 hematological toxicity=73%	11695811
International Federation of Gynecology and Obstetrics study on stage II to IV epithelial ovarian cancer patients (n=59)	Response rate (complete response + partial response)=72%	Quality-of-life scores improved significantly during therapy due to modest amount of toxicities.	11324769
Prospective randomized trials (phase I/II trials) by the Gynecologic Oncology Group and the European Organization for Research and Treatment of Cancer	No differences in efficacy for carboplatin/paclitaxel compared to cisplatin/paclitaxel arm	Reduce toxicity for carboplatin/paclitaxel compared to cisplatin/paclitaxel arm.	10190787
Advanced stage EOC patients (n=50)	Response rate=72%; Recurrence or progression of disease=68%		9514799
International Federation of Gynecology and Obstetrics study on Advanced epithelial ovarian cancer stages IIC, III, and IV (n=90)	patients receiving carboplatin/cisplatin + paclitaxel had a high overall response (82%).	Grade 3 and 4 neutropenia=20%; Grade 3 and 4 thrombocytopenia=4% for carboplatin group. Grade 3 and 4 neutropenia=32%; Grade 3 and 4 thrombocytopenia=7%, for the cisplatin arm	9346224
GINECO, a French cooperative clinical trials group for gynaecological cancer research (multicenter phase II carboplatin/paclitaxel study): (n=50).	Overall response rate=43%.	Grades 3 and 4 neutropenia=30%; Transitory peripheral neuropathy=45%.	9346219
Phase I study of carboplatin/paclitaxel in patients with stage IIC to IV ovarian cancer: (n=30)	Overall response rate=57%.	Grades 1 and 2 peripheral neurotoxicity=53%; patients developed Grade 4 neutropenia=31%.	9045330

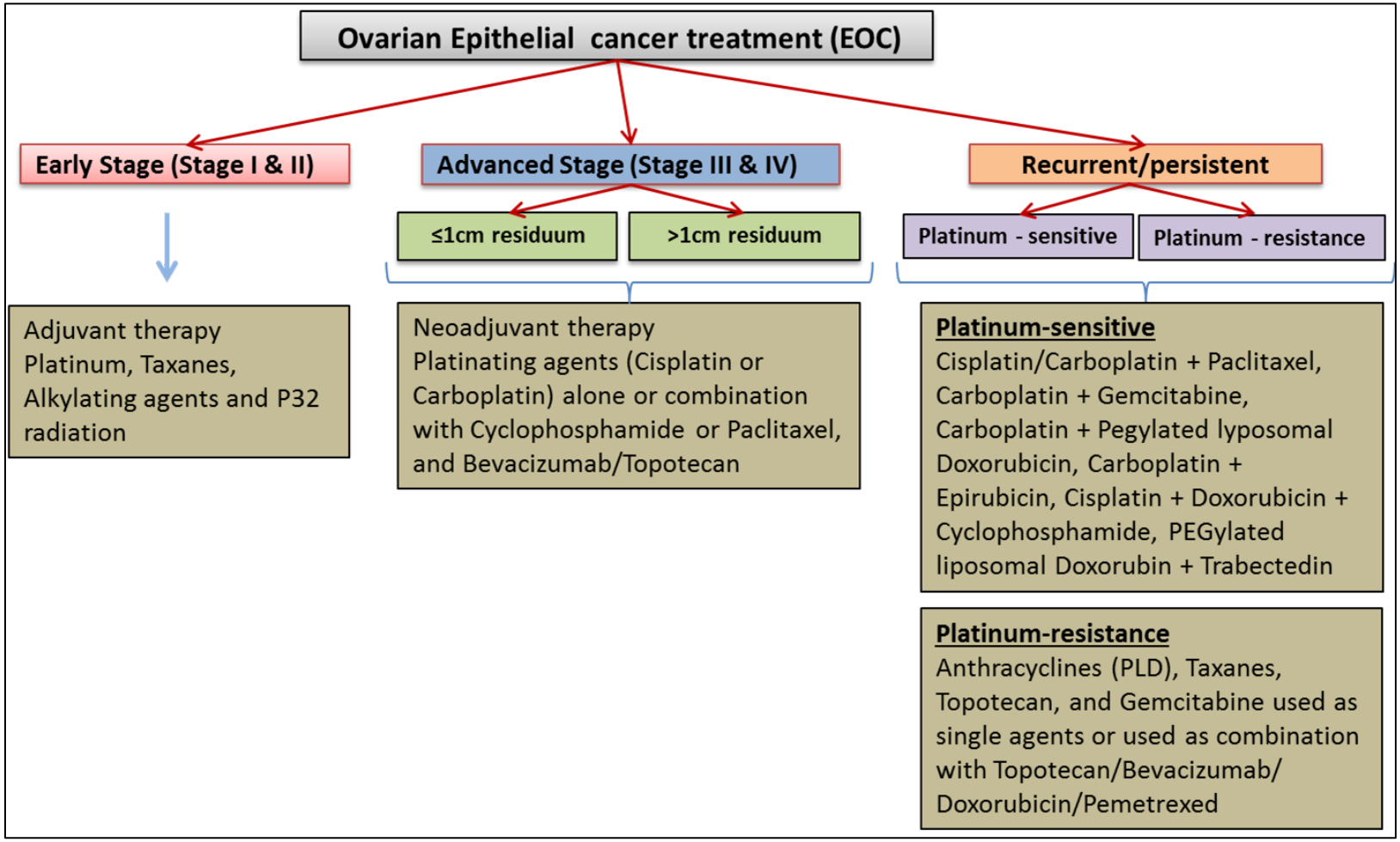


Figure 1.2 Overall treatment strategies for epithelial ovarian cancer (EOC)

1.2 Lung cancer: Non-small cell lung cancer (NSCLC)

1.2.1 Introduction

In addition to ovarian cancer, platinum agents and paclitaxel are also used to treat patients diagnosed with lung cancers. This section is a brief overview of non-small cell lung cancer (NSCLC).

Lung carcinoma is the most common malignancy worldwide, and the leading cause of cancer deaths. According to the most recent estimates, the global incidence of lung cancer is more than 1.6 million cases/year, resulting in more than 1.3 million deaths/year (>18% of all cancer deaths) [74]. In the USA, the incidence of lung cancer was estimated to be 221,200 cases in 2013, leading to nearly 158,040 deaths (27.5% of all cancer deaths) [3]. Five-year relative survival with for lung cancer patient was 15.7% (from 1995 to 2001) [75]. Lung cancer can either arise directly from lung tissue or as a result of metastasis from other parts of the body. Broadly, there are two main types of primary tumor described as either small-cell or non-small-cell lung carcinomas **(Figure 1.3**).

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer constituting around 80% of lung cancers. It is histologically heterogeneous and constitutes squamous cell carcinoma, large cell carcinoma and adenocarcinoma (based on data obtained from National Cancer Institute-Non-small cell lung cancer treatment). NSCLC are therefore classified by the World Health Organization (WHO)/International Association Lung Cancer (IASLC) based on histology. There are three main subtypes, i) squamous cell carcinoma (25% of lung cancers), ii) adenocarcinoma (40% of lung cancers) and iii) large cell carcinoma (10% of lung cancers). Squamous cell carcinoma is located centrally, in the larger bronchi of the lung and strongly related with smoking. Squamous cell carcinoma is further subdivided into papillary, clear cell, small cell and basaloid tumor based upon histology of tumor. Similarly, adenocarcinoma is also subdivided into acinar, papillary tumor,

solid adenocarcinoma with mucin, adenocarcinoma with mixed subtypes and bronchioloalveolar carcinoma. Adenocarcinoma has the frequent histologic heterogeneity.

The 5-year relative survival of patients with advanced NSCLC remains approximately 4 % [76]. One major drawback of lung cancer is that the majority of lung cancer patients are diagnosed in advanced stages based on small biopsy and cytology [77]. Determination of stage is very important for prognosis and selection of therapy. NSCLC staging is based on histology of tumor, resection margins (surgical margin) of it and also lymph node involvement (status and location of lymph node). Based upon these NSCLC stage is determine by TNM classification according to American Joint Committee on Cancer (AJCC) 2010, whereas T = size and extension of tumor, N = lymph node involvement and M = extension of metastasis [78].

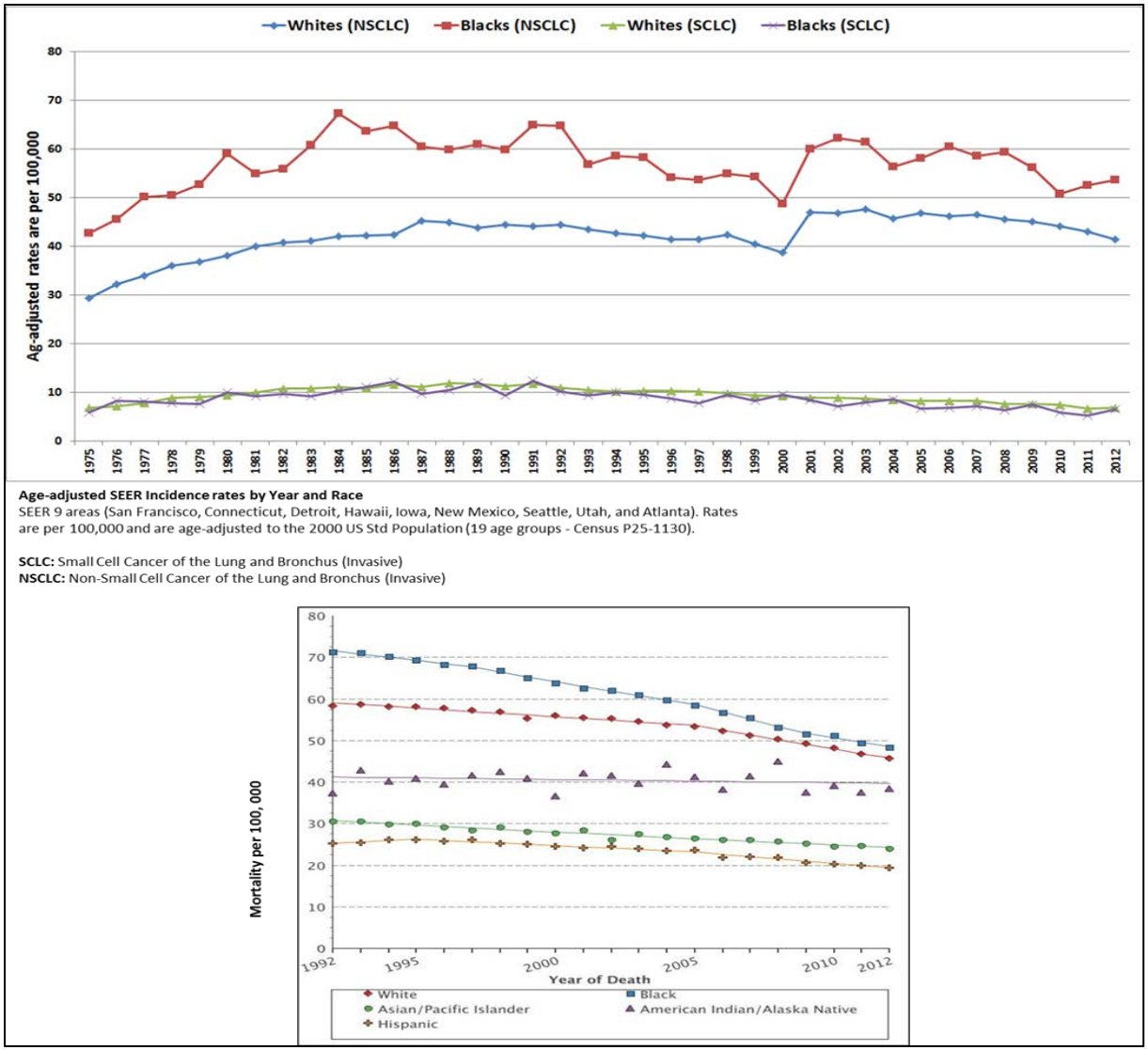


Figure 1.3 Incidence and Mortality of lung cancer; Non-small cell lung cancer (NSCLC) (NCI SEER cancer statistics).

1.2.2 Risk Factors

Several risk factors are associated with development of lung cancer. Major risk factors include cigarette, pipe, or cigar smoking. The risk for lung cancer is on average tenfold higher in smokers compare to nonsmokers. The risk increases with the quantity of cigarettes, duration of smoking, and starting age [79]. Exposure to second-hand smoke, radon, arsenic, asbestos, chromates, chloromethyl ethers, nickel, polycyclic aromatic hydrocarbons, radon and air pollution are also considered as potential risk factor of lung cancer [80].

1.2.3 Symptoms and Diagnosis

The most common symptom is worsening cough or chest pain. Other presenting symptoms are hemoptysis (coughing up blood), malais (discomfort, illness), weight loss, dyspnea/ breathlessness, hoarseness (abnormal voice change). Symptoms from distant metastases can show neurological defect or personality changes from brain metastases or pain from bone metastases. Physical examination also shows supraclavicular lymphadenopathy, pleural effusion or lobar collapse, unresolved pneumonia, chronic obstructive pulmonary disease or pulmonary fibrosis [81].

1.2.4 Prognostic factors

Prognosis of disease depends on current physical status, current disease status with stage, and prior physical status. Depending on these three prognostic factors alone, median survival varies between 6 weeks and over a year. Additionally, other factors such as tumor size, histologic type, and cell type, age and sex appeared to be important when considered alone. Adverse prognosis is related with large tumor size >3 cm, histology-nonsquamous, vascular invasion and if tumor metastasize in lymph nodes. Prognosis is adversely affected by poor performance status and weight loss of more than 10% in inoperable condition patients [82-87].

Biomarkers including genetic mutations and gene expression are important prognostic factors for lung cancer or Non-Small Cell Lung Cancer. These include identification of significant mutations

in patient groups and strategies to molecular targeted therapy are therefore important in improving the survival of these subsets of patients [88]. Relevant genes include epidermal growth factor receptor (EGFR), Kirstein rat sarcoma viral oncogene (KRAS), Human epidermal growth factor receptor2 (HER2), V-raf murine sarcoma viral oncogene homolog B1 (BRAF), PI3K catalytic protein alpha (PI3KCA), gene rearrangements in anaplastic lymphoma kinase (ALK) as well as somatic mutations and gene duplications in MAP2K1/MEK, a hepatocyte growth factor receptor, [89-91].

1.2.5 Treatment of Non-Small cell lung cancer (NSCLC)

Treatment of NSCLCs depends on staging (as shown in **Figure 1.4).** Standard treatment options are surgery, chemotherapy and radiation therapy. Platinum-based chemotherapy is the standard for NSCLC [92]. Commonly use drug regimens are cisplatin/etoposide (EP5050) or weekly carboplatin/paclitaxel [93-95]. Additionally, paclitaxel is also used as combination with cisplatin or carboplatin [92, 96, 96, 97, 97-110]. Platinum-based radiation chemotherapy (Platinum + etoposide or cisplatin + mitomycin + vindesine) is also used as a treatment option and may improve survival of patients with locally advanced NSCLC. However, toxicity is also high for combination therapy than single therapy [100, 111-115]. Many clinical trials have shown Cisplatin-based combinations plus radiation therapy shows 10% reduction in the risk of death compared with radiation therapy alone [116-119]. Maintenance therapy with single agent or initial chemotherapy is recommended for patients with stable or responding disease after four cycles of platinum-based combination chemotherapy [101, 120, 121].

In spite of these treatment options, 5-year survival rate of NSCLC is very poor (3% to 7%) for majority of patients [122, 123].

1.2.6 NSCLC clinical trials using Platinum/Paclitaxel combination therapy

Carboplatin (PC) plus paclitaxel (Taxol) is a standard treatment of advanced non-small-cell lung cancer (NSCLC). In **Table 1.2** we evaluate the results from clinical trials performed on non-small-

cell lung cancer (NSCLC) patients using combination of paclitaxel (Taxanes) and carboplatin Chemotherapy. Similar to our observation regarding clinical trials in ovarian cancers, variations in drug response and drug-related toxicities are major cause of concerns for patients diagnosed with NSCLC undergoing platinum/paclitaxel-based combination chemotherapy. Understanding the genomics and transcriptomics governing these inter-individual variations in treatment outcome may provide tools to guide treatment and tailor therapies specific to each patient with the goal of achieving maximum therapeutic effects.

Table 1.2 Summary of clinical trials of carboplatin/paclitaxel combination therapy in NSCLC.

Patient details	Drug response/outcome	Drug Toxicity	PubMed ID
Patients with stage IIIb-stage IV NSCLC (n=90)	Overall response=40%. One-year survival=50.7%	Grades 3/4 leukopenia=13.3%,; anemia=15.5%; thrombocytopenia= 11.1%.	11863090
Stage IIIB or IV NSCLC (n=100) patients.	Objective response=36%,.	Grade 3/4 peripheral neuropathy=18%.	9007133
Advanced stage NSCLC patients (n=33).	Complete responses=41%; partial responses=41%.	Grade 3/4 esophagitis=51%.	9007133
Phase II trial at the the Fox Chase Cancer Center and its network on patients with advanced NSCLC	Response rate =55%; median event-free survival=24 weeks; median survival=47 weeks,	Peripheral sensory neuropathy=75%; myelosuppression=44% (grade 4 granulocytopenia 38% and grade >=3 thrombocytopenia 50%-70%).	9331128
Phase II trial conducted by the Eastern Cooperative Oncology Group with stage IV non-small cell lung cancer (NSCLC).	Overall response rate=27%. Median survival=38 weeks; Survival rate at 1-year=32%	Grade 3 or 4 granulocytopenia=47%; Thrombocytopenia=3%. Most common non-hematologic toxicities were nausea, emesis, neuropathy, arthralgia and myalgia.	9007120
Phase II trial in patients with advanced NSCLC (n=54)	Response rate= 50%.	Myelosuppression was observed as principal toxicity. Grade 3 or 4 granulocytopenia=70%; thrombocytopenia=13%; anemia=9%; fatigue=9%; hemorrhagic cystitis=1% after the first cycle.	7543559
Multi-center study of two dose levels of paclitaxel and carboplatin in locally advanced and metastatic non-small cell lung cancer (NSCLC) (n=130, 99 in the high dose (HD) and 31 in the low dose (LD) cohort)	Overall best clinical response rate =23.8%; one complete (CR); 30 partial responses (PR). One-year survival=34%.	Myelosuppression was the most prominent side-effect for both cohorts. Grade 3-4 leucopenia =HD 34.4% and LD 19.3%; neutropenia (HD 59.2, LD51.6%. The most prominent non-hematologic toxicities were alopecia and polyneuropathy.	11396237

Stage	Treatment	5-yr Survival
IA & IB	1) Surgery (Lung tumor and lymph node surgery) 2) Surgery not approved or declined - Radiation therapy 3) Chemotherapy if cancer still present(IB) 4) Drugs: Carboplatin, Paclitaxel	45-49%
IIA & IIB	1) Surgery → adjuvant chemotherapy +/- radiation (Chemoradiation therapy) 2) Surgery not approved or decline - Radiation / Chemoradiation therapy 3) Drugs: carboplatin, Paclitaxel	30%
IIIA (resectable)	1) Surgery → adjuvant chemotherapy /Chemoradiation therapy 2) Surgery not approved or declined - Chemoradiation therapy 3) Drugs: Carboplatin, Paclitaxel	14%
IIIB & IV (unresectable)	1) Radiation / Chemoradiation (Stage III) 2) Chemotherapy or Targeted therapy (Tyrosine-kinase inhibitor-TKI).	1-5%

Figure 1.4 NSCLC Treatment Summary by Stage (NCCN guidelines; Version 1.2016).

1.3 Platinum drugs

1.3.1 Introduction

This section provides discussion of the key agents that are the focus of this thesis. Platinum drugs are currently used to treat various types of cancers including ovarian, non-small cell lung cancer (NSCLC), testicular cancer, lymphomas and myelomas. **Figure 1.5** shows the major members of this class of platinating agents.

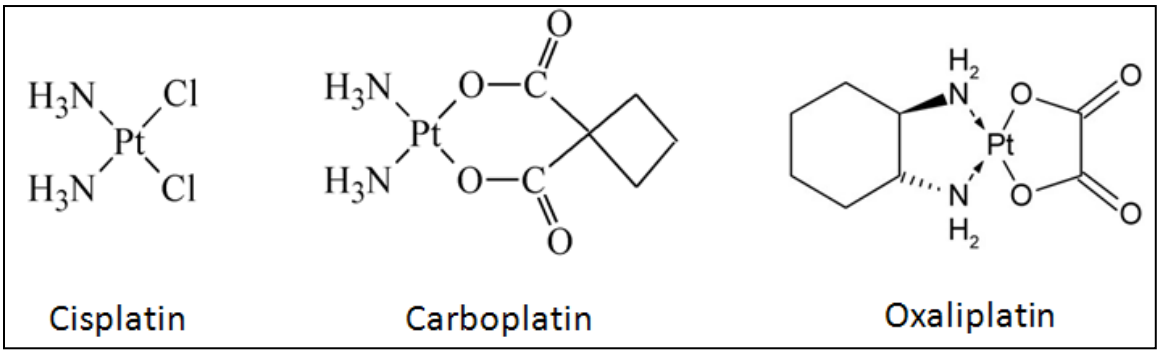


Figure 1.5 Chemical structure of Platinum drugs

Cisplatin was the first platinum drug to be approved by FDA. Therapeutic potential of cisplatin was discovered by Barnett Rosenberg in 1969[130]. Unfortunately, cisplatin chemotherapy has dose-limiting side effects including nephrotoxicity, ototoxicity, myelosuppression and neurotoxicity [131]. To overcome cisplatin toxicity, the second generation platinating agent carboplatin (Diammine[1,1-cyclobutanedicarboxylato(2-) -O,O'] platinum(II); Empirical formula: $C_6H_{12}N_2O_4Pt$; Molar mass: 371.249 g/mol; Trade name: Paraplatin) was developed and approved by the FDA in 1989 for the treatment of advanced ovarian cancer [132]. Additionally, to reduce toxicity carboplatin is administrated by a 30-minute intravenous infusion of 300 to 500 mg/m2 based on a patient renal function according to Calvert Formula:

[Total Dose (mg) = (target AUC) x (GFR + 25)].

Neurologic toxicity due to carboplatin based therapy is low/very rare (~ 5%).

However, although carboplatin is routinely used for treatment of ovarian and other cancers, one of the biggest challenges faced by clinicians is the variation in clinical response, [133, 134] and toxicities including myelosuppression (Thrombocytopenia ~ 25%,Neutropenia ~ 16%, leukopenia ~ 16%, Anemia ~ 71%), gastrointestinal toxicity (vomiting, nausea and diarrhea ~ 65%,) [133] as well as development of drug resistance [134]. Therefore, a large number of the cancer patients relapse after first line based chemotherapy due to wide inter-patient variation [131, 135].

Differential expression and/or activity of genes involved in the platinum drug pathway due to presence of single nucleotide polymorphisms (SNPs) may have an impact on treatment outcome and toxicity in cancer patients being treated with platinum compound. Here we discuss the most relevant genetic variations in the genes involved in the platinum-drug pathway and to evaluate their association with chemotherapy.

1.3.2 Mechanism of action of Platinum drugs

Platinum drugs are highly polar hydrophilic molecules. They do not easily diffuse across hydrophobic/lipophilic lipid membranes. Therefore, following intravenous administration of carboplatin or cisplatin, they remain inactive in their native form in the high chloride environment of plasma [131]. Upon transport of platinating agents by ion pumps and transporters, they enter the cell where, owing to a much lower concentration, the carboxyl ligands are slowly displaced by water (aquation), at the rate of 10^{13} molecules/sec, leading to the development of aqua ligands. The positively charged aqua ligands are then displaced and the platinum atom binds to purine bases on DNA forming DNA adducts as mono adducts, 1,2-intrastrand DNA cross-links (1-2GpG, 1-3GpG, 1-2GpA) and inter-strand crosslinks. These DNA crosslinks are attributed to the formation of DNA helix-distorting adducts that result in strand breakage activating DNA repair mechanisms that interfere with cell division, which ultimately results in apoptosis [131]. The mechanism of action of platinum drugs is shown in **Figure 1.6**.

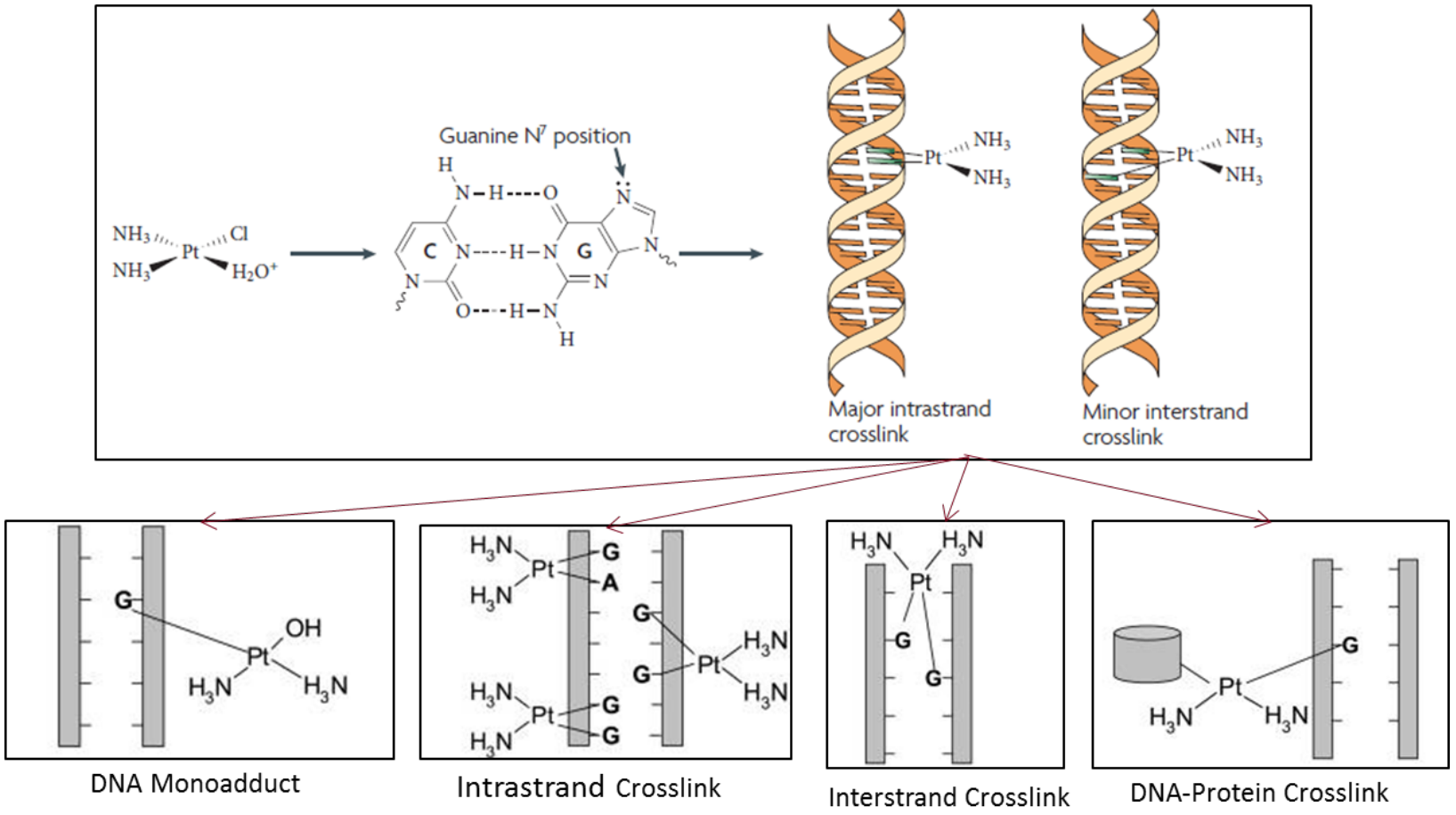


Figure 1.6 Mechanism of action of platinum drugs.

Upon entering the cell platinum drugs lose chloride or oxalate ions, and gains two water molecules. The platinum atom binds to N^7 of purine in the DNA forming DNA adducts as mon-adducts and through 1,2-**intra-strand** (involving adjacent guanines on the same strand) and **inter-strand** (involving binding to guanines on opposite DNA strands).

1.3.3 Metabolic pathway of platinum drugs

1.3.3.1 Genes of importance in drug pharmacokinetics (PK)

Figure 1.7 shows detailed metabolic pathway of platinum drugs including the pharmacokinetics (PK) and the pharmacodynamics (PD).

Platinum drugs enter the cell as a "passenger" along with normal copper transport by binding to the N- terminus of Copper Transporter 1 (CTR1) followed by CTR1 monomer crosslinks without blocking copper binding [136, 137]. The Copper Transporter 1 (CTR1), encoded by the gene Solute Carrier Family 31(SLC31), thus plays an important role in the influx of platinum drugs into the cell [138-140]. It is a 28kDa protein consisting of 190 amino acids. It has 3 trans-membrane domains. Mammals and yeasts lacking CTR1 have been shown to have lower accumulation of platinum inside the cell. A study demonstrated that the loss of CTR1 function is associated with 10-15% decrease in cisplatin entering the cell resulting in a two fold increase in cell survival [141]. Low or reduced expression of CTR1 has been correlated with resistance to carboplatin and cisplatin and overexpression has been associated with increased carboplatin and cisplatin uptake [142-146]. Furthermore, studies have also shown that CTR1-knockout results in resistance to platinum drugs. CTR1 lacking cells accumulates 50% less cisplatin and eightfold higher resistance compare to the CTR1+ cells [147]. CTR1-/- tumor xenografts in mouse models also are not responsive to platinum drugs. Loss of CTR1 decreases the platinum drug binding to the transporter and reduces influx of cisplatin by 81%, oxaliplatin 68% and for carboplatin almost complete elimination of [148]influx (Larson et al. 2009). However, once inside the cell, platinum drug also initiates the rapid degradation of CTR1 ion through ubiquitination and proteosomal action mediated by copper chaperone antioxidant protein (ATOX1). Proteasome inhibitors like bortezomib have been shown to block this process of CTR1 degradation which leads to increase in the uptake and transport of platinum-containing drugs. The molecular mechanisms of platinum compound involved

endocytosis of CTR1is not completely clear yet [148, 149]. Some evidences show that CTR2 is involved in platinum compound/drug accumulation and that its expression correlates with the sensitivity of ovarian carcinoma cell lines to cisplatin [139, 150]. Additionally SLC21A8 expression was significantly associated with 3 platinumbased chemotherapeutics agents such as cisplatin, carboplatin, and (trans-l-1,2-diaminocyclo-hexane)platinum-II (DACH-Pt), which is structurally related to the another platinating agent oxaliplatin ($p= 0.025$) [151].

Copper transporting ATPase (ATP7A and ATP7B) provides efflux and resistance to platinum containing drugs along with copper transport [152-155]. Cisplatin and other platinum drugs like carboplatin and oxaliplatin molecules bind to the first four metal N-terminal binding sites of ATPase copper transporter beta (ATP7B) transporter and stimulate catalytic phosphorylation of ATP7B, which eventually facilitated drug efflux [140, 155-157]. ATP7B transfection in human epidermoid carcinoma cell line KB-3-1 resulted in resistance to both cisplatin and carboplatin [158, 159]. *In vivo* studies in breast carcinoma [160] and ovarian cancer cell lines [161, 162] have shown association of expression of these genes with cisplatin and/or carboplatin resistance. ATP7B is the primary candidate for platinum drug resistance where ATPase copper transporter alpha (ATP7A) play minor role. ATP7B knockdown ovarian cells show increase cisplatin sensitivity and increased efficacy with cisplatin therapy. However, the mechanism between this association is not completely clear. High ATP7B gene expression is significantly associated with cisplatin resistance in ovarian carcinoma cell lines. In 2008/MNK ovarian cancer cell lines, increased ATP7A expression is predictive of poor survival ($p<0.00057$) to the cisplatin, carboplatin and Oxaliplatin [154]. Additionally, in ovarian cancer cell line model ATP7B genes were expressed at higher levels in platinum-resistant cells compared with sensitive cells. ATP7B silencing showed 2.5-fold reduction of cisplatin IC_{50}, increased DNA adduct formation and reducing tumor growth along with increased tumor cell apoptosis, and reduced angiogenesis [152]. However, overexpression of ATP7A and ATP7B in Me32a fibroblast resulted in increased resistance to cisplatin but not to carboplatin

[163]. Homologous transporters ATP7A and B are 160-170-KDa membrane proteins with eight transmembrane and several cytosolic domains. The n-terminal cytosolic domain contain six copper binding sites where as ATP binding domain is located between transmembrane domain six and seven [164, 165]. ATP7A and ATP7B are primarily involved in the copper transport from cytoplasm into trans-Golgi network. As the copper content of the cell increases, ATP7A moves from trans-Golgi network to the plasma membrane while ATP7B relocates to intracellular vesicular compartments, to facilitate the export pathway [166, 167].

ATP-Binding Cassette (ABC) transporter superfamily of proteins with 48 members are among most ancient and largest protein families since they have been found present in all living organisms, from prokaryotes to humans. Classification of proteins as ABC transporters is based on the sequence and organization of their ATP-binding cassette (ABC) domains [168-170]. Glutathione conjugates of platinum compound can be excreted from the cells by members of the ABCC transporter family [171]. Therefore, the most important ABC transporters studied in context of transport of platinum-containing drugs are ABCC2 and ABCG2 – which are very frequently associated with resistance of cancer cell to multiple drugs, also known as multidrug resistance (MDR) [172]. ABCC2 - ATP-Binding Cassette, Sub-Family C (CFTR), Member 2 or Multidrug Resistance- Protein 2 (MRP2) and ABCG2 - ATP-Binding Cassette, Sub-Family G (WHITE), Member 2 are members of ATP-binding cassette (ABC) transporter superfamily that regulate secretion of organic anions and efflux of platinum compounds [138]. Cytotoxicity of cisplatin, carboplatin and oxaliplatin was significantly lowered in MDCKII cells transfected with ABCG2 transporter. The IC(50) values in MDCKII-ABCG2 were 25.7 for cisplatin, 164 for carboplatin and 165 micro M for oxaliplatin whereas IC(50) for MDCKII cells were 15.4 for cisplatin, 133 for carboplatin and 50.3 micro M for oxaliplatin [173]. Cisplatin-resistant melanoma cells showed a distinct overexpression of MRP2/ABCC2 on mRNA and protein level [174]. Platinum drug resistance Ovarian carcinoma line A2780RCIS, the adrenocortical carcinoma line D43/86RCIS and the melanoma line MeWoCIS1

overexpressed of MRP2/ABCC2. The level of DNA platination corresponded inversely to the level of MRP2 expression in these cell lines. Therefore, functional inhibition of MRP2 might be a promising treatment strategy for resistance to platinum-based anticancer drugs [175]. Multidrug resistance-associated protein 1 (MRP1) is a protein which is encoded by the ABCC1 gene and it play an interesting role in platinum drug resistance through the GS-X pump. The GS-X pump actively effluxes the glutathione S-platinum (GS-Pt) complex to detoxifying cells [176]. Additionally, ABCC4 overexpression significantly associated with decreased cisplatin sensitivity in lung cancer patients [138, 171].

Several genes, such as Myeloperoxidase (MPO), Superoxide Dismutase 1 (SOD1), GSTM1, NAD(P)H Quinone Dehydrogenase 1 (NQO1), GSTP1, and MT are responsible for lowering the intracellular concentration of platinum drugs. Increased concentrations of Glutathione (GSH), Metallothioneins (MT) induce resistance against cisplatin. Cytosolic glutathione S-transferase (GST) supergene family includes genes belonging to the subfamilies [alpha] (GSTA), μ (GSTM), [pi] (GSTP) and [theta] (GSTT). GSTs are phase II metabolic isozymes involved in detoxification of many xenobiotic agents like anticancer drugs and carcinogens through conjugation with reduced glutathione (GSH) [177]. This class of enzymes detoxifies carcinogens, therapeutic drugs, and environmental toxins by conjugation with glutathione. Genetic variations in GST genes can affect toxicity and efficacy of drugs. In ovarian tumor cell lines Glutathione S-transferase P1 knockdown showed 2.3- and 4.83-fold change in cisplatin and carboplatin chemosensitivity (IC_{50}) respectively [178-180]. On the other hand, Glutathione S-transferase P1 knockdown in mesothelioma cells exhibited a decrease in GST enzyme activity and GSTπ protein levels along with an increase in both glutathione levels and sensitivity to cisplatin and oxaliplatin [181]. Similarly, GST-pi mRNA was significantly increased in cisplatin resistant gastric cancer and bladder cancer cells [182, 183]. SOD is involved in superoxide generation and plays an important role in Platinum drugs detoxification in

cell. Inhibition of the SOD1 activity has been shown to enhanced the cisplatin sensitivity in the ovarian cancer resistant cells [184].

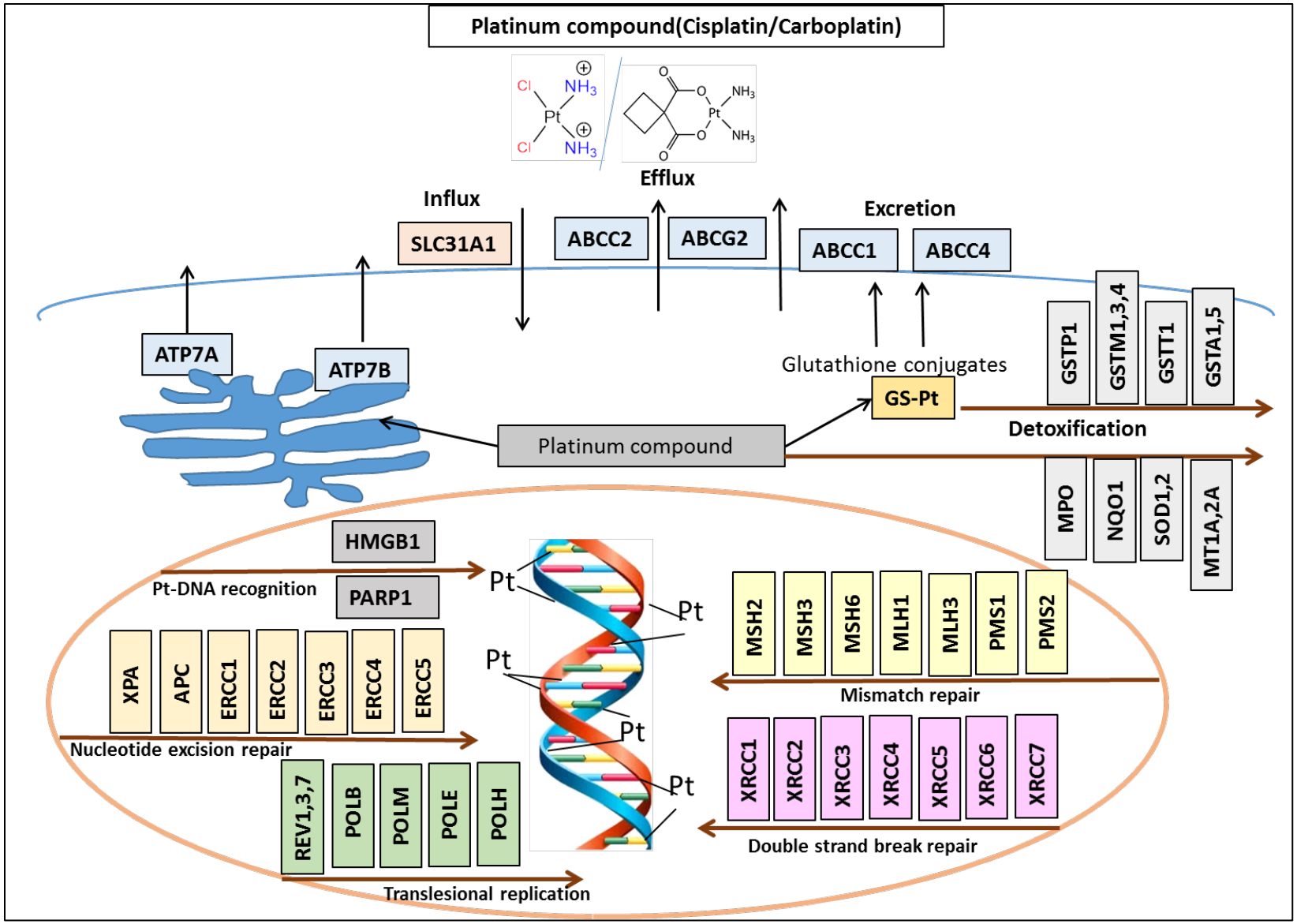


Figure 1.7 Metabolic pathway of Platinum drugs

1.3.3.2 Genes of importance in drug pharmacodynamics (PD)

1.3.3.2.1 Pt-adduct repair pathway

Upon entering the cell, platinum drug binds to DNA and forms adducts [185]. High mobility group (HMG) proteins recognize and bind to DNA intra-strand crosslinks and prevent replicative bypass repair and nucleotide excision repair (NER) leading to initiation of apoptosis [131, 186]. HMGB1 thus inhibits DNA repair and causes more cisplatin DNA damage in murine model. Knocking out HMGB1 in cell lines showed increased DNA repair ([187]. Expression levels of HMGB1 have also been significantly associated with the potency of cisplatin and carboplatin in cancer cells [188]. Binding of HMGB1 to DNA-Pt adducts is very specific and the amount of binding is correlated with cytotoxicity of platinum compounds [185]. HMGB2 have been shown to enhance the cisplatin sensitivity of cells by inhibiting repair of the DNA lesion caused by cisplatin. Additionally, Polymerase 1 (PARP1) is a protein that also recognizes DNA lesions and plays an important role in the activation of the Base excision repair (BER) pathway [189-193].

1.3.3.2.2 Mismatch repair pathway

Single stranded DNA damage due to Pt-DNA adducts are recognized and repaired by the DNA mismatch repair pathway (**Figure 1.8**) [194]. Genes involved in the mismatch repair pathway include MutS Homolog 2 (MSH2), MutS Homolog 3 (MSH3), MutS Homolog 6(MSH6), MutL Homolog 1(MLH1), MutL Homolog 3 (MLH3), Postmeiotic Segregation Increased 2 (PMS2), Exonuclease1 and PCNA. Lack of detection of Pt-DNA adducts by the mismatch repair genes MSH, MLH and PMS2 (MSH2, MSH3, MSH6, MLH1, MLH3, PMS1 and PMS2) may fail to trigger apoptosis which result in resistance to the platinum drugs [194, 195]. A number of *in vitro* studies have suggested that MMR gene expression deficiency is a cause of platinum resistance. Furthermore, loss of hMLH1 in ovarian tumor cells was found to be correlated with cisplatin resistance [196]. The human colon cancer cell line HCT116+ch2 showed 2.1-fold resistance to cisplatin and 1.3-fold resistance

to carboplatin compared to the subline expressing a wild-type copy of hMLH1. Concurrently, human endometrial cancer cell line HECS9 deficient in hMSH2 showed 1.8-fold resistance to cisplatin and 1.5-fold resistance to carboplatin when compared to a subline with wild-type hMSH2 [197]. Another study reported that hMLH1 mRNA and protein expression levels were significantly decreased in the cisplatin-resistant head and neck squamous cell carcinoma cell lines compared with a cisplatin-sensitive cell line [198]. Furthermore, dMSH2 knockout embryonic stem cell line was 2.1-fold more resistant to cisplatin and 1.7-fold more resistant to carboplatin when compared with wild-type wt-2 cell line while the PMS2-/- mouse fibroblasts were 1.9-fold more resistant to cisplatin and 1.5-fold more resistant to carboplatin when compared to the isogenic PMS2+/+ fibroblasts [199]. MSH3 deficiency increase sensitivity to both cisplatin and oxaliplatin at clinically relevant doses. MSH3 deficiency contributes to the cytotoxicity of platinum drugs through deficient DSB repair [200]. Another study reported that, loss of MLH1, MLH2, MSH2, MSH3, MSH6 in isogenic strains of Saccharomyces cerevisiae led to increased resistance to cisplatin and carboplatin. Loss of PMS2 gene was found to be associated with an increased sensitivity, ranging from 2-6-fold to platinum compounds cisplatin and oxaliplatin in another study [201].

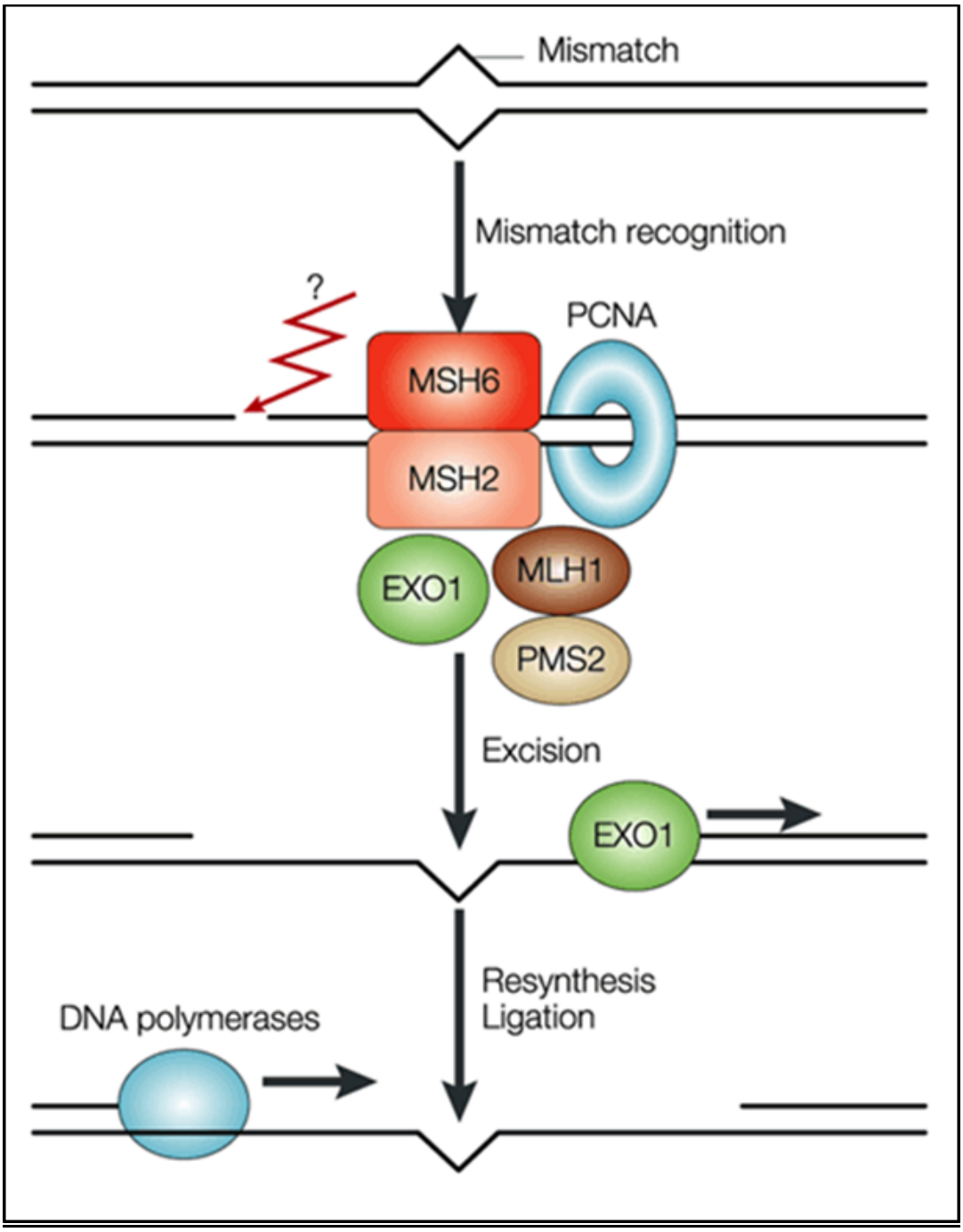


Figure 1.8 Mismatch repair (MMR) pathway.

The MMR pathway recognizes and repairs single stranded DNA damage due to Pt-DNA adducts. Major genes involved in this pathway include the MutS and MutL Homolog genes, PMS2, Exonuclease1 and PCNA.

1.3.3.2.3 Nucleotide excision repair pathway

The nucleoside excision repair (NER) pathway genes Xeroderma pigmentosum group (XPA), XPC, XPB/ Excision repair cross-complementing group 1 (ERCC3), XPD (ERCC2), XPG (ERCC5), XPF (ERCC4), ERCC1, DNA polymerases and DNA ligases are primarily involved in the identification and repair of platinum-DNA adducts (1, 2-intrastrand DNA cross-links) [202] **(Figure 1.9)**. The human platinum-resistant ovarian carcinoma cell line SKOV3/DDP showed expression levels of ERCC1 were significantly suppressed after stably transfecting with shERCC1-recombinant plasmid, whereas increased sensitivity to cisplatin ($p<0.01$). In another study siRNA-mediated ERCC1 silencing in the MCF-7, HeLa S3 and HCT116 cells showed both the ERCC1 mRNA and protein expression were significantly inhibited, which led to a decrease in repair activity of cisplatin-induced DNA damage along with cell viability against platinum-based drugs such as cisplatin, carboplatin, and oxaliplatin [203]. On the other hand, ovarian cancer, which is frequently more resistant to cisplatin, shows increased expression of NER proteins including ERCC1 and XPC [204]. XPA promotes resistance in tumor cells to platinum based chemotherapeutic drugs. XPA was expressed at higher levels in cisplatin-resistant melanoma cells than in cisplatin-sensitive ones. Furthermore, the knockdown of XPA not only increased cellular apoptosis and also inhibited cisplatin-induced autophagy, which increase cisplatin sensitivity. XPA promoted cell-protective autophagy by polymerase 1 (PARP1) activation in a DNA repair-independent manner and cell became cisplatin resistance [205]. Additionally, expression of XPA was higher in NSCLC tissues than that in normal lung tissues. Furthermore, silencing XPA gene increased the apoptosis and cisplatin sensitivity [206].

XPB mRNAs have been observed to be expressed >2-fold higher in clear cell epithelial ovarian tumors [207]. Higher mRNA levels of XPB were observed in clinically resistant tumor to platinum-based chemotherapy. In a cohort of 27 patients, mRNA levels of XPB was 5-fold higher in platinum-resistant tumors ($P = 0.001$). Furthermore, these platinum-resistant tumors also show

higher mRNA levels of ERCC1 and XPA [208]. ERCC1 is an excision nuclease within the nucleotide excision repair pathway that forms a heterodimer with XPF. ERCC1-XPF complex inhibitor reduced the level of this protein in ovarian cancer cells, inhibited Nucleotide Excision Repair and sensitized cells to cisplatin [209]. Non-small cell lung cancer, ovarian and breast cancer cell lines showed increased cisplatin cytotoxicity and efficacy when ERCC1/XPF was down-regulated by RNAi [210]. The expression of XPF was upregulated in cisplatin resistance KB carcinoma cells (KCP-4) [211]. Osteosarcoma cells (U2OS) by XPF and XPG knockdown exhibited increased sensitivity to platinum drugs compared to the parental cells up to fivefold [212]. XPF is also required for the repair of DNA lesions through the Nucleotide Excision Repair and Interstrand Crosslink Repair pathways. Xeroderma pigmentosum D (XPD) protein expression levels may also influence response to platinum-based chemotherapy in epithelial ovarian cancer (EOC). In resistance group of serous ovarian cancer, XPD protein expression levels were significantly higher than sensitivity group. Upregulation of ERCC1 and XPD protein expression was associated with resistance process to platinum-based chemotherapy in advanced EOC [213]. XPD expression was significantly related to resistance to platinum-containing treatment ($p = 0.043$) [214].

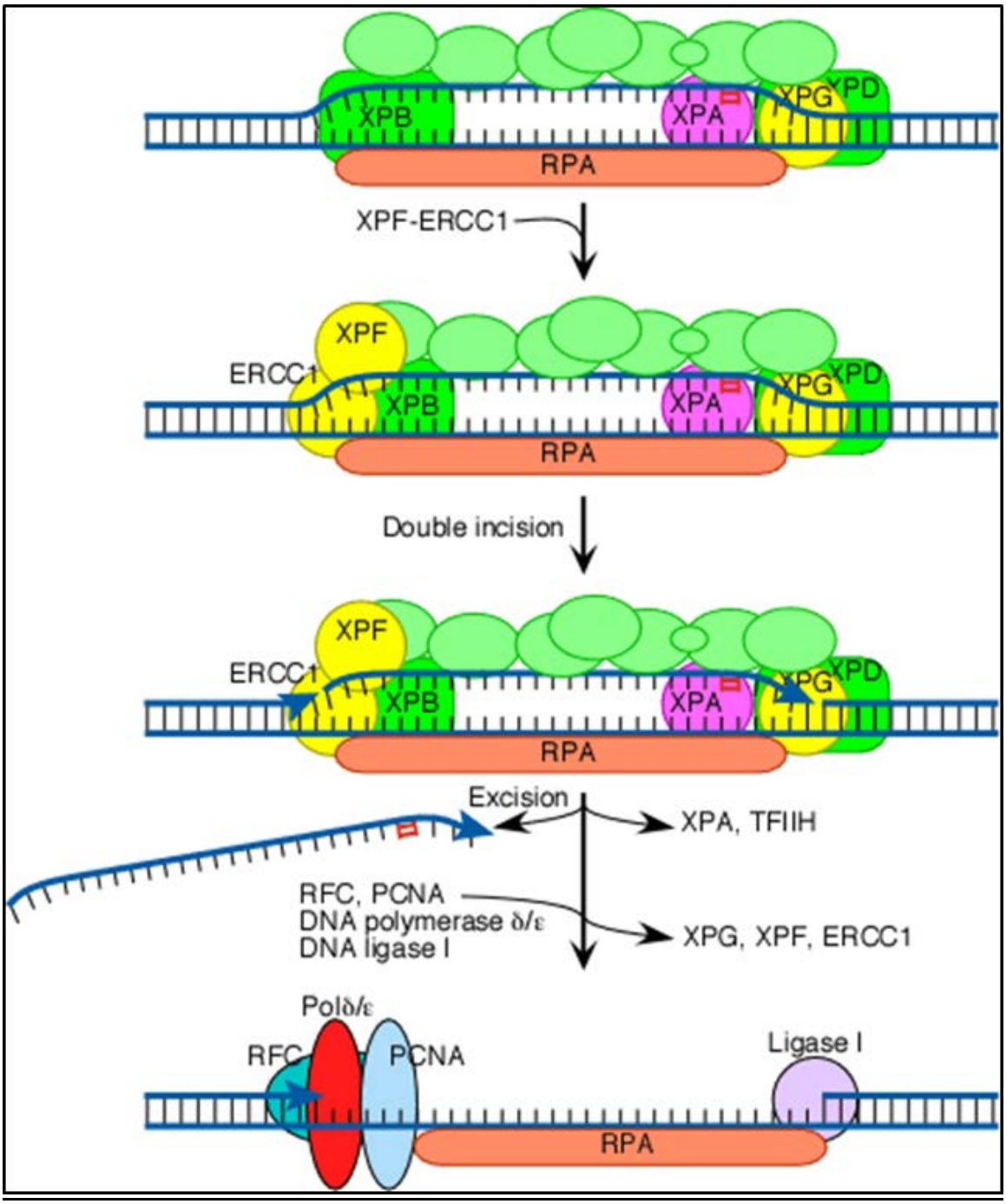


Figure 1.9 Nucleotide excision repair (NER) pathway

The NER pathway is involved in the identification and repair of platinum-DNA adducts (1, 2-intrastrand DNA cross-links). Major genes involved in this pathway include the Xeroderma pigmentosum group genes A-G, DNA polymerases and DNA ligases.

1.3.3.2.4 Homologous recombination and non-homologous end joining

Homologous recombination and non-homologous end joining (NHEJ) DNA repair processes are also involved in the repair of double-stranded breaks due to DNA-Pt adducts (Thacker and Zdzienicka. 2004). The genes XPF and ERCC1 gene are involved in homologous recombination while X-ray repair cross complementing group/ X-Ray Repair Complementing Defective Repair in Chinese Hamster Cells 1 (XRCC1), XRCC2, XRCC3, XRCC4, XRCC5, XRCC6 and XRCC7 are involved in non-homologous end joining process [215]. Protein expression of XRCC1 was significantly increased in cisplatin-resistant gastric cancer cells and caused cisplatin resistance to the cells. Furthermore, Inhibition of XRCC1 expression increased sensitivity of resistant cells to cisplatin [216]. High XRCC1 expression was found to be associated with platinum resistance ($p < 0.0001$) in ovarian cancer [217].

1.3.3.2.5 Translesional bypass replication repair

Finally, when all DNA repair mechanisms fail, translesional bypass replication or SOS repair pathway is activated to overhaul the DNA damages by pt-DNA adducts. Several genes including Polymerase (DNA Directed), Beta or POLB (DNA pol β), POLH (DNA pol η), POLK, POLM, DNA polymerase zeta, REV1, REV3 and REV7 are involved in this pathway [218]. DNA pol β and DNA pol η Overexpression lead to cisplatin resistance. Polymorphism of these genes also associated with clinical response to the platinum drugs in cancer patients. REV3 is an essential component for of Polζ and both play an important role in SOS repair mechanism. Reduced levels of Rev3 exhibited sensitivity to cisplatin and significantly associated with better OS of platinum drug treated mice [219]. Expression of hRev3 mRNA in the cisplatin resistant head and neck squamous cell carcinoma cell line was approximately 1.4-fold higher than in the sensitive cells. Furthermore, hRev3 knockdown with siRNAs increases sensitivity to cisplatin in this cell line [198]. Reduced expression of the DNA polymerase zeta (catalytic subunit REV3) made fibroblasts more sensitive to cisplatin [218, 220].

1.3.4 Pharmacogenomics of platinum drug pathway genes

Pharmacogenomics is a field of research that combines pharmacology and genomics to study the effect of genes and genetic variations on the response to drugs with an ultimate goal of developing safe and efficacious 'best-fit' medications based on an individual's genetic make-up [221].

We performed a comprehensive review of the research on pharmacogenomics of platinum drug pathway genes conducted through a systematic search of papers reporting association between gene expression and/or genetic polymorphisms in Platinum drug pathway genes and clinical response to platinum-drug based therapy. The genes included in the ensuing review are the most important genes that have been reported to be involved in the transport (influx and efflux), DNA adducts repair, detoxification/xenometabolism (PK) and PD of platinum-based drugs. The criteria for identifying the genes to be selected for the study was based on the information available from the resources providing information on key pathways, e.g. KEGG (Kyoto Encyclopedia of Genes and Genomes), GO (NCBI's Gene Ontology database), PharmGKB (The Pharmacogenomics Knowledgebase) and PGRN's (Pharmacogenomics Research Network) drug-specific pathway information. Care was taken to ensure uniformity with respect to gene names and SNP IDs based on scientifically accepted notations and nomenclatures. The following sections will be divided into studies conducted on genetic variations within genes involved in platinum drug pharmacokinetics (transporters, drug metabolizing enzymes) and pharmacodynamics including a summary of the major studies and the conflicting evidences, if any.

1.3.4.1 Genes involved in transport and metabolism of platinating agents (PK genes)

1.3.4.1.1 Drug efflux and influx Transporter

CTR1/SLC31 (Chromosomal location 9q32; length 42965bp; 5 exons) is a predictor of platinum-based chemotherapy response and also plays a role as prognostic factor in NSCLC [222, 223]. Ctr1 is predictor of platinum-based chemotherapy response and also plays a role as prognostic factor in

NSCLC [223]. On the other hand, high SLC31A1/CTR1 expression of was associated with poor survival of (HNSCC) head and neck squamous cell carcinoma patients [224]. In African Americans CTR1 expression is significantly reduced (P=0.001) along with tissue platinum concentrations (P=0.009) and tumor shrinkage (P=0.016) compared to Caucasians [225]. Additionally, CTR1 expression is a predictive/prognostic factor for a good outcome in stage III endometrial cancer patients treated with adjuvant carboplatin based combination chemotherapy. The patients with tumors showing high expression levels of CTR1 associated with longer DFS (P = 0.058) and significantly longer OS (P = 0.003) [226]. Another recent study showed CTR1 expression was significantly associated with improved chemotherapeutic responses. The median survival time was 15 months in patients with low CTR1 expression, but >66 months in patients with high CTR1 expression (P<0.001)[227].

Table 1.3 summarizes genotype-phenotype associations between polymorphisms within platinum drug influx and efflux transporter genes and treatment outcome of platinating agent-containing cancer chemotherapy.

The CTR1 SNP rs10981694 A>C was associated with cisplatin ototoxicity in NSCLC patients. C-Variants are poor tolerance to ototoxicity (p, 0.05) [228]. Genetic polymorphisms rs7851395 A>G and rs12686377 T>G were significantly associated with platinum resistance in NSCLC patients treated with platinum-based anticancer drugs. Patients with a GT haplotype showed increased susceptibility to platinum resistance (P < 0.05), whereas an AG haplotype contributed to longer survival (P < 0.05) in Chinese NSCLC patients {{}}. Further, deletion of the CTR2 gene leads to increased cisplatin and carboplatin accumulation in cells [150].

Genomic Location for ATP7A gene is Chromosome Xq21.1 and it is 139,740 bases long with 23 exons and 3 splice variants. ATP7B is located at chromosome 13q14.3. This gene is 121,014 bases in length with 34 exons. ATP7A and ATP7B play important roles in platinum drugs efflux.

Resistance cell line to carboplatin, nedaplatin and oxaliplatin showed up-regulated expression of ATP7A, ATP7B transporter [229]. In NSCLC patient's low ATP7A expression was associated with an improved prognosis. The median survival time was 20 months in patients possessing tumors with high ATP7A expression, but >66 months in patients possessing tumors with low ATP7A expression (P<0.001). ATP7B expression was also significantly associated with tumor cell differentiation [227]. ATP7B mRNA and protein expression in colorectal tumors is associated with clinical outcome to oxaliplatin/5FU. Patients with the lowest ATP7B expression significantly associated with longer time to progression (p = 0.0009) and increased risk of progression (p = 0.002) along with the maximum benefit from platinum based therapy [153]. ATP7A-positive patients NSCLC patient showed significantly poorer histological grade (p = 0.039) and poorer response to platinum-based chemotherapy (p = 0.001) compared with ATP7A-negative patients. Therefore, ATP7A expression may be used as an independent prognostic factor for OS (p = 0.045) for NSCLC patients treated with platinum based therapy [230]. In a recent gene profiling study (Taqman low-density array-TLDA) on cisplatin sensitive cell lines showed ATP7B gene expression was decreased 1.95-fold (p=0.03) but there was no change in gene expression of ATP7A or SLC31A1 [231].

Only one study so far has investigated the association of ATP7a and ATP7B transporter SNPs with platinum drug response by sequencing of these genes in Japanese cancer patients treated with oxaliplatin/5-fluorouracil/leucovorin and paclitaxel/carboplatin. 38 genetic variations, including 30 novel ones (seven nonsynonymous) in ATP7A and 28 novel and 33 known genetic variations (including 13 nonsynonymous SNPs) in ATP7B were detected These SNPs possibly influence the efficacy and toxicities of oxaliplatin and carboplatin treatment by changing the drug concentrations within tumor cells [232].

The ABCC2 gene is located on chromosome 10 (10q24.2) and it is 69,979 bases long. This gene has 34 exons with 3 splice variants; whereas ABCG2 or Breast cancer resistance protein (BCPR)

is located on chromosome 4 (4q22.1) and 141,154 bases long with 19 exons and 4 splice variants. ABCC2 and ABCG2 are involved in carboplatin efflux [138, 233]. Multiple studies have shown association of these transporters with survival in ovarian cancer patients. Low ABCC2 gene expression in tumor tissues from patients with ovarian cancer was associated with prolonged Progression Free Survival (PFS)[233]. Additionally, localization of ABCC2 in the nuclear membrane was associated with cisplatin resistance and clinical outcome in ovarian carcinoma and overexpression of ABCC2 at mRNA and protein levels was shown to be associated with decreased formation of intra-strand DNA cross-links and resistance to cisplatin in melanoma cells [234]. In gastric cancer ABCG2 over-expression was shown associated with longer OS for platinum-containing chemotherapy for [235]. In HNSCC (head and neck squamous cell carcinoma) patient's expression of ABCC2, and ABCG2 indicates poor survival of patients treated with platinum based therapy. High ABCB1, ABCC1 expression was associated with both favorable progression-free survival (PFS, p = 0.0357, p = 0.0183) and OS (p = 0.0535. p = 0.038) [224].

The non-synonymous polymorphisms rs2273697 (1249G>A/Val417Ile) in ABCC2 and rs2231142 (C421A/Gln141Lys) in ABCG2 were correlated with treatment response and survival in ovarian cancer patients treated with platinum-based chemotherapy. SNP rs2231142 was associated with longer median PFS [236]. Another study reported, polymorphism ABCG2 rs2231142 (C421A/ Q141K) significantly associated with worse OS for lung cancer patients treated with platinum-based drugs (HR: 1.60; 95% CI 1.04-2.47; n = 256) [237]. ABCG2 SNPs rs2725264 and rs4148149 were associated with OS (P=0.041, P= 0,014) in 129 NSCLC patients treated with first-line platinum-based chemotherapy. The median survival duration for the rs2725264 T/T, T/C, and C/C genotypes was 35.75 , 34.25 and 14.89 respectively[238]. The ABCC2 polymorphism, rs8187710 (4544G>A), is associated with OS (p=0.036) in advanced NSCLC patients treated with platinum-based therapy. Allele A was associated with adverse OS (p=0.009) of IV NSCLC patients [239]. Polymorphisms in ABCC2 rs717620 (C-24T) and rs3740066 (C3972T) was associated with

treatment response. Homozygotes of -24C was associated with a better treatment response (P=0.032). Whereas patients with 3972T had increased risk of severe thrombocytopenia toxicity with female patient group (P=0.034), with overall toxicity (P=0.02) and hematologic toxicity (P=0.002) among platinum-treated non-small cell lung cancer patients [240, 241].

ATP-Binding Cassette, Sub-Family B (MDR/TAP) Member 1 (MDR1/ABCB1) is a 209,691 bases long gene located at chromosome 7q21.12. The MDR1/ABCB1 synonymous coding polymorphism rs1045642 (C3435T/Ile1145Ile) was associated with platinum-based chemotherapy response in lung cancer where the C/C genotype was more sensitive to platinum-based chemotherapy than patients with C/T and T/T. Frequency of this SNP is highest in Asian and Caucasians populations and the lowest in African populations [242-246]. rs2032582 (G2677T) is a nonsynonymous SNP located in exon 21 of the ABCB1 gene. ABCB1 2677G>T/A polymorphism were significantly associated with grade 3 or 4 hematological and gastrointestinal toxicities in epithelial ovarian cancer patients and in non-small-cell lung cancer patients treated with platinum and taxen compounds [247, 248]. Variant allele (T) of the ABCB1 SNP rs1128503 was associated with the risk of anemia in ovarian cancer patients treated with platinum compound. (C > T; p = 0.023, OR = 1.71, 95% CI = 1.07-2.71) [249].

ABCC1 gene is located at chromosome 16p13.11 (length: 193,498 bases) and ABCC4 is located at chromosome 13q32.1 (length: 281,618 bases). Higher mRNA expression of ABCC1 significantly correlate with short period of progression free survival in advanced ovarian carcinoma patients treated with platinum based chemotherapy [250]. rs1729786, a tagSNP in the gene ABCC4 was found significantly associated (OR=0.68; 95% CI=0.50–0.92; unadjusted p=0.037) with chemotherapy-induced peripheral neuropathy (CIPN) among 950 lung cancer patients treated with platinum based therapy [251].

Table 1.3 Summary of SNPs in drug transporter genes associated with response to platinating agents.

SNP ID	Gene symbol	Gene Function	Cancer Type	Associated Phenotype	Associated Genotype	PubMed ID (s)
rs10981694	SLC31	Influx transporter	NSCLC	Cisplatin ototoxicity	rs10981694 (A>C) C Variants show poor tolerance to ototoxicity	22516052
rs7851395, rs12686377	SLC31	Influx transporter	NSCLC	Platinum resistance, survival	AG haplotype of rs7851395 A>G and rs12686377 T>G contributed to longer survival. Patients with GT haplotype show increased susceptibility to platinum resistance	22725681
rs2273697	ABCC2	Efflux transporter	Ovarian cancer	Treatment response, survival	1249G>A or Val417Ile	22112610
rs2231142	ABCG2	Efflux transporter	Ovarian cancer	Median PFS	C421A or Gln141Lys	22112610
			Lung cancer	OS	rs2231142 significantly associated with worse OS (HR: 1.60; 95% CI 1.04-2.47)	19107936
rs2725264, rs4148149	ABCG2	Efflux transporter	NSCLC	OS	rs2725264 vs OS (P=0.041); rs4148149 vs OS (P= 0.014)	23689644
rs8187710, rs717620	ABCC2	Efflux transporter	NSCLC	OS , treatment response	rs8187710 vs OS (p=0.036). Allele A was associated with adverse OS (p=0.009); rs717620 (C-24T), 24CC was associated with a better treatment response (P=0.032).	26816351, 19568750
rs3740066	ABCC2	Efflux transporter	NSCLC	Toxicity	rs3740066 (C3972T) 3972T had increased risk of severe thrombocytopenia toxicity (P=0.034), overall toxicity (P=0.02) and hematologic toxicity (P=0.002)	20943283

rs1045642	ABCB1	Efflux transporter	Lung cancer	Chemosensitivity	rs1045642 (C3435T) C/C genotype more sensitive to platinum-based chemotherapy than patients with C/T and T/T.	24687344, 22932088, 22766400, 22704851, 22296372
rs2032582	ABCB1	Efflux transporter	Ovarian cancer	Toxicity	rs2032582 (G2677T), vs grade 3 or 4 hematological and gastrointestinal toxicities	19203783, 20189873
rs1128503	ABCB1	Efflux transporter	Ovarian cancer	Anemia	C > T; p = 0.023, OR = 1.71, 95% CI = 1.07-2.71	25881102
rs1729786	ABCC4	Efflux transporter	Lung cancer	CIPN	rs1729786 vs Chemotherapy-induced peripheral neuropathy (p=0.0377)	25586538

1.3.4.1.2 Xenobiotic metabolism genes

Polymorphisms in GST genes are associated both with cancer susceptibility and anticancer drug resistance [252]. GSTP1 gene is located on chromosome 11 (11q13.2). This gene has 7 exons along with 7 splice variants. Low GSTP1 expression level was found to be associated with better response in NSCLC patients (P <0.003) [253]. Polymorphisms in GST genes are associated both with cancer susceptibility and anticancer drugs resistance [252]. Genetic variations in GST genes including rs1695 (GSTP1 I105V, shown in **Figure 1.10**) have been shown to be associated both with resistance, toxicity and efficacy of platinum-based drugs [247, 252, 254, 255]. rs1695 (GSTP1 Ile105Val, A313G) was associated with clinical out come in NSCLC patients. GSTP Val/Val exhibited a shorter survival time, and had a 1.89-fold greater risk of death than patients with the IIe/IIe genotype. Similar association was also reported by several studies [241, 256-263]. Additionally, Patients with homozygous mutant GSTP1 GG genotype were at risk for severe platinum-associated polyneuropathy (P = 0.01) in NSCLC platients [255]. rs1695 is also associated with neutropenia in NSCLC patients. Toxicity was less for patients with the 105Val allele (p = 0.020) [264]. Similarly, rs1695 was also found to be associated with PFS (p = 0.004) in ovarian cancer patients [265, 266]. rs1695 was also found to be a significant risk factor for grade 3 or 4 hematological (adjusted OR, 3.08; 95% CI, 1.12-8.43) and gastrointestinal toxicities (adjusted OR, 9.74; 95% CI, and 1.59-15.85) [267]. Additionally, high expression of GSTP1-1 was significantly associated with a poor DFS and OS (P = 0.047 and P = 0.033, respectively) [234], as well as poor PFS in ovarian cancer patients [268]. Furthermore, the GSTP1-A-allele was also associated with better prognosis (P = 0.032) and GSTP1-AG genotype was associated with necrosis in the tumor's post-chemotherapy histology (P = 0.001) testicular germ cell tumor [269]. GSTP1 expression and rs1695 GA/GG was also significant associated with poor OS (P=0.004) in gastric cancer in Chinese population [270-272]. rs1695 was also found to be contributed in colorectal cancer patients treated with oxaliplatin. Patients with Val/Val genotypes significantly associated with better and longer survival of colorectal cancer [273, 274]. rs1695 was also associated

with toxicity for cisplatin based radiochemotherapy (P = 0.005) [275] esophageal cancer. Furthermore, rs1695 was also contributed in oxaliplatin-related neuropathy. Patients with homozygous GSTP1 105Ile allele showed more toxicity than patients homozygous or heterozygous for the GSTP1 105Val allele (P = 0.02) in gastrointestinal cancer patients treated with oxaliplatin-based chemotherapy [276]. Another SNP rs1138272 (Ala114Val) was found to be associated with platinum based chemotherapy treated NSCLC patients. Genotype (Ala/Val or Val/Val) had significantly better survival compared with patients with (Ala/Ala; P = 0.037). Median survival was 16.1 months compare to 11.4 months for Ala/Ala [277].

Glutathione S-Transferase Mu 1(GSTM1) gene is located on chromosome one (1p13.3). The most important polymorphism in GSTM1 is a partial gene deletion in GSTM1 gene which is called GSTM1 null genotype, resulting in complete absence of GST M1 enzyme activity. 5-year survival and time to progression was better for GSTM1-null genotype patients compared with GSTM1-wt genotype patients (P = 0.001 and P = 0.013, respectively) in ovarian cancer patients receiving platinum based chemotherapy. GSTM1-wt genotype was also important predictors of risk of death (P = 0.036) [278]. Additionally, thrombocytopenia, anemia and neuropathy were less among patients with the GSTM1-null or GSTM3 intron 6 AGG/AGG genotype in ovarian cancer patients receiving platinum based therapy [265, 279]. GSTM1 null polymorphism was also associated with treatment response in testicular germ cell tumors, NSCLC patients and lung cancer patients where GSTM1-null were superior responders to platinum drugs than GSTM1-wt (P = 0.014)[258, 261, 269, 280].

Glutathione S-Transferase Theta 1 (GSTT1) is located on chromosome 22 (22q11.23) and 8,179 bases long. GSTT1 overexpression was found to be influenced decreased cisplatin sensitivity in lung cancer patients treated with platinum compound ([171]). Non-null GSTT1 genotype was a poor prognostic factor for overall response in ovarian cancer [247, 281] and toxicity [247, 266, 279]. Similar association was also observed in gastric cancer patients where response rate was higher in GSTT1 (+) genotype compared with GSTT1 (-) genotype [271].

Glutathione S-Transferase Alpha 1 (GSTA1) gene is located on chromosome 6 (6p12.2). The GSTA1 promoter region polymorphism rs3957357 (-69 C>T) polymorphism correlated with the OS in ovarian cancer patients receiving platinum drugs chemotherapy. Patients with T/T genotype survived longer than C/C carriers (P=0.044) [265]. GSTA1∗B polymorphism comprise with -52G>A (rs3957356), −69C>T (rs3957357) and -567T>G (rs4715332) were also associated with an increased OS in patients with different malignancies treated by platinum compound[282].

Glutathione S-Transferase Mu 3 (GSTM3), Glutathione S-Transferase Mu 4 (GSTM4) and Glutathione S-Transferase Mu 5 (GSTA5) genes are located on chromosome 1. The GSTM3 SNP rs1799735 or GSTM3*B induces high GSTM3 expression and results in beneficial effects in cisplatin chemotherapy [265, 266, 283] in ovarian cancer. Two tag SNPs in GSTA5 (rs4715354) and GSTM4 (rs560018) were associated with survival in lung cancer patients treated with platinum based drugs. Furthermore, rs560018 was significantly associated with IC50 in lymphoblastoid cell lines generated from 100 healthy Caucasian-American subjects (p=0.019) [171].

SOD1 and SOD2 are also involved in platinum drug xenometabolism. The SOD2 mis-sense/nonsynonymous coding SNP rs4880 (47T/C or Val16Ala) CT + CC combined genotype was shown to be significantly associated with decreased median OS time of 23 months when compared to the TT genotype (P=0.002) in gastric cancer patients receiving platinum based combination therapy [270]. SNP rs4880 was also found to be associated with ototoxicity in cisplatin treated pediatric medulloblastoma patients Texas Children's Cancer Center (1987-2010). The rs4880 T > C substitution results in a Val > Ala amino acid change at position 16 of the SOD. The C-allele of the rs4880 variant was significantly associated with increased SOD2 activity which leads to ototoxicity (odds ratio = 3.06, 95% confidence interval: 1.30-7.20) [284].

Myeloperoxidase (MPO) is another component of the detoxification pathway of platinum drugs. The mutant genotypes of the MPO promoter SNP -463G>A was shown associated (A allele) with lower carboplatin-induced hematological toxicity in cancer patients [285]. Another missense SNP

rs1800566 (C609T/Pro187Ser) in the gene NAD(P)H Quinone Dehydrogenase 1 (NQO1) was significantly associated with higher prevalence of TT genotype ($P < 0.001$). TT genotype carriers had less chance to response with chemotherapy than compared to CC genotype carriers ($P = 0.003$) in advanced NSCLC receiving platinum-based chemotherapy [286]. **Table 1.4** lists the salient variations in xenobiotic metabolism genes associated with pharmacogenomics of platinum drug-based chemotherapy.

Table 1.4 Summary of SNPs in drug metabolism genes associated with Platinum-based chemotherapy.

SNP ID	Gene symbol	Cancer Type	Associated Phenotype	Associated Genotype	PubMed ID (s)
rs1695	GSTP1	NSCLC	Clinical outcome, polyneuropathy	rs1695 (GSTP1 Ile105Val, A313G) Val/Val exhibited a shorter survival time, and had a 1.89-fold greater risk of death than patients with the IIe/IIe genotype. Patients with GG genotype were at risk for severe platinum-associated polyneuropathy (P = 0.01)	023167352, 24958519, 24729086, 22761669, 22009704, 21766492, 19568750, 17409936, 11844594, 22031394, 17409936
		Ovarian cancer	PFS, Toxicity	rs1695 vs PFS (p = 0.004); rs1695 vs grade 3 or 4 hematological (adjusted OR, 3.08; 95% CI, 1.12-8.43); vs gastrointestinal toxicities (adjusted OR, 9.74; 95% CI, and 1.59-15.85)	22188361, 19786980, 19203783
		Testicular germ cell tumor	Prognosis	A allele was associated with better prognosis (P = 0.032) and AG genotype was associated with necrosis in the tumor's post-chemotherapy histology (P = 0.001)	19741569
		Gastric and Colorectal cancers	Tumor response, PFS, and OS	Patients with Val/Val genotypes significantly associated with better and longer survival.	27154175, 25901207, 23020798, 9626464, 22994779, 19922504,
		Esophageal cancer	Toxicity	Rs1695 vs toxicity for cisplatin-based radiochemotherapy (P = 0.005)	21286719
		Gastrointestinal cancer	Neuropathy	Ile/Ile showed more toxicity than Val/Val and Val/Ile genotype (P = 0.02) in patients treated with oxaliplatin-based chemotherapy	16707601
rs1138272	GSTP1	NSCLC	Survival	Genotype (Ala/Val or Val/Val) had significantly better survival compared with patients with (Ala/Ala) (P = 0.037).	16342067

rs3957357	GSTA1	Ovarian cancer	OS	Patients with rs3957357 (C>T) T/T genotype survived longer than C/C carriers (P=0.044) .	19786980
rs4880	SOD2	Gastric cancer, medulloblastoma	OS	rs4880 CT/CC was significantly associated with decreased median OS time compared to the TT genotype (P=0.002).	25901207, 26400460
MPO-463G>A	MPO		Toxicity	Promoter SNP -463G>A was associated with lower carboplatin-induced hematological toxicity	24083736
rs1800566	NQO1	NSCLC	Response	rs1800566 TT genotype carriers showed less response compared to CC genotype carriers (P = 0.003)	24464627

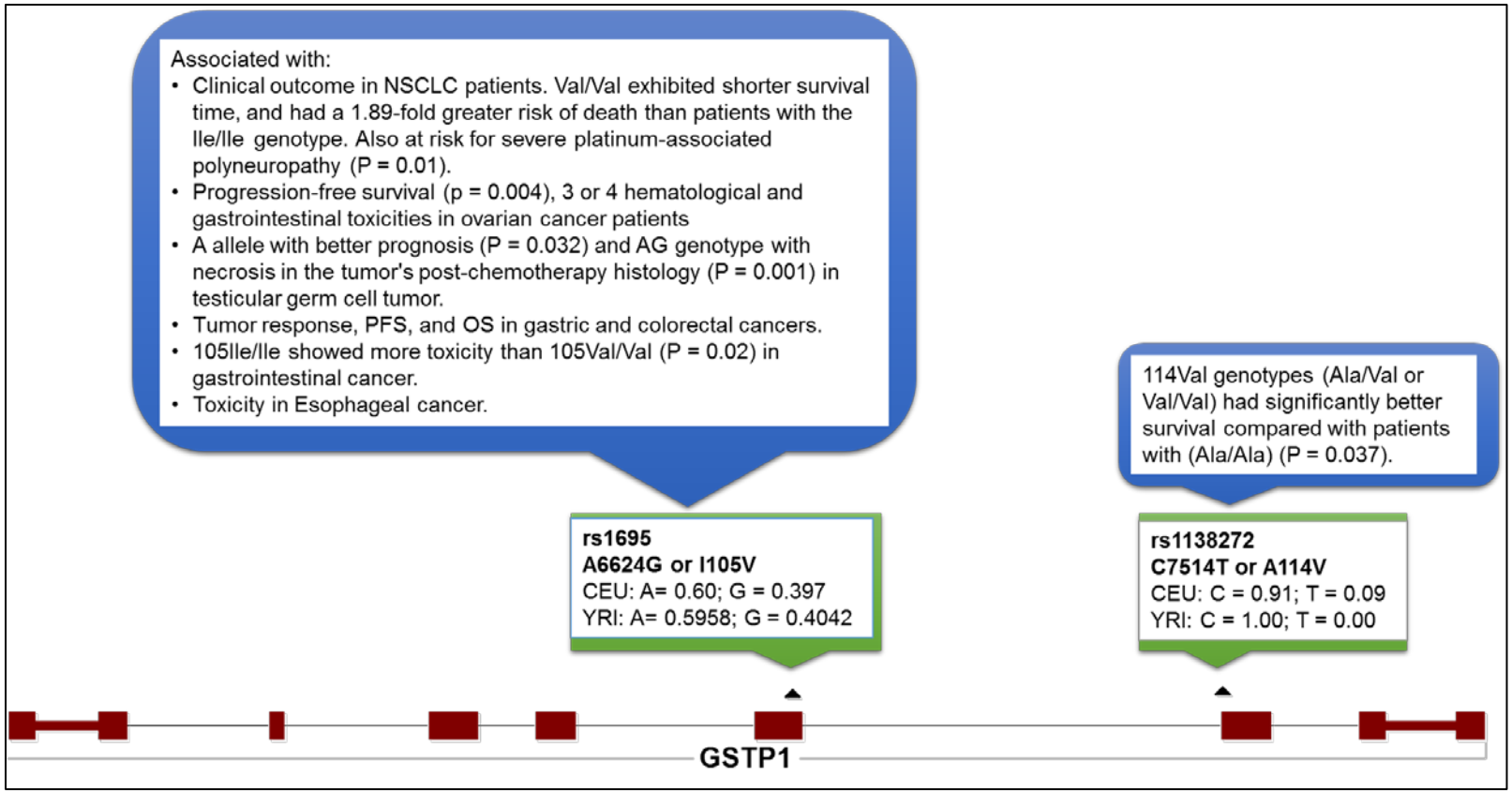


Figure 1.10 Genetic variants in GSTP1 associated with survival/outcome and/or toxicity in patients treated with Platinum drug-based chemotherapy (Green boxes denote coding SNPs).

1.3.4.2 Genes involved in DNA damage and repair (PD) pathway and their impact on platinating agent response

Several genes are involved in the drug pharmacodynamics pathway. HMGB and PARP are involved in the identification of pt-DNA adducts which is followed by repair of these DNA damages (pt-DNA adducts) through NER repair genes, Mismatch repair genes, double strand break repair genes and finally translesional replication repair (SOS) pathway genes when all other repair pathway fails. Polymorphisms in these DNA repair genes may modulate not only DNA repair capacity, but also clinical outcome of treatment with DNA damage-inducing anticancer drugs including platinating agents **(Tables 1.5 and 1.6)**.

1.3.4.2.1 Pt-DNA adduct repair

The HMGB1 gene is located at chromosome 13q12.3. It is 160,894 bases long including 8 exons and 8 splice variants. In epithelial ovarian cancer (EOC) patients treated with platinum based therapy HMGB1 expression was an independent predictor for PFS (P = 0.024). High HMGB1 expression associated significantly with poorer prognosis (PFS) [287]. In Chinese lung cancer patients, rs1412125 and rs2249825 of HMGB1 were significantly associated with the platinum-based chemotherapy response [288]. Poly (ADP-Ribose) Polymerase 1 (PARP1) is a 47,410 bases long gene; located in chromosome 1q42.12 with 23 exons and 10 splice variants. PARP1 is a 113 kDa protein that contains a DNA-binding domain with two zinc fingers and an auto-modification domain. The non-synonymous polymorphism in PARP1 rs1805412 (Val762Ala/T2285C) influenced the prognosis of advanced NSCLC patients treated with platinum-based chemotherapy. PARP1 2285CC genotype was a significantly associated with poor PFS (CC vs. CT/TT: adjusted HR = 1.90, 95 % CI = 1.02-3.52) [289].

1.3.4.2.2 Mismatch repair pathway genes

MSH2 and MSH6 are located at chromosome 2p21 and 2p16.3 respectively, whereas MSH3 is located at chromosome 5q14.1. Downregulation of hHSH2 expression has been shown associated with improved survival, while high expression was correlated with poor survival in ovarian cancer patients following cisplatin treatment [154]. Furthermore, MSH2 expression was associated with a shorter time to tumor recurrence, resistance to chemotherapy and death in testicular cancer [290]. On the other hand, human MSH6 (hMSH6) defects result in 1.5-4.8 fold increase in cisplatin resistance and 2.5-6 fold increase in replicative bypass of cisplatin adducts [291]. MSH6 protein levels were also correlated to progression during Platinum-based chemotherapy (P = .0281) in patients with malignant pleural mesothelioma (MPM) [292].

Among the mismatch repair gene polymorphisms evaluated for correlation of with response to cisplatin- or carboplatin-based chemotherapy, gIVS12-6T/C (hMSH2) was associated with treatment response (p=0.0173) in peripheral lymphocytes from advanced NSCLC patients belonging to the Chinese population [293]. Significant correlation was found between the MSH3 5'UTR SNP rs1105524 and PFS. The G/A and A/A genotypes (median survival time: 14.27 months; 95%CI = 9.80-18.75) showed shorter survival than patients with the G/G genotype (median survival time: 26.37 months; 95%CI = 15.03-37.71) (P = 0.04) [293].

MLH1 is located at chromosome 3p22.2 with 21 exons while MLH3 is located at chromosome 14q24.3 and it has 14 exons. Decreased hMLH1 expression was significantly correlated with improved survival, while high expression was correlated with poor survival in ovarian cancer patients following cisplatin treatment [294]. A study by Scartozzi et al observed that among 38 patients with advanced ovarian cancer treated with cisplatin-based chemotherapy, 19 (56%) showing loss of hMLH1 expression with a median survival of 55 months while 15 (44%) showing normal hMLH1 expression had a lower median survival of 12 months (P=0.014) [295]. MLH1 expression

was also significantly associated with PFS (P = .0205) following platinum-based chemotherapy in malignant pleural mesothelioma[292]. In addition, MLH1 expression was associated with a shorter time to tumor recurrence, resistance to chemotherapy and death in testicular cancer [290]. Non-functional hMLH1 caused 1.5-4.8-fold increased cisplatin resistance and 2.5-6-fold increased replicative bypass of cisplatin adducts [291]. The non-synonymous coding MLH1 polymorphism rs1799977 (Ile219Leu/A655G) was shown associated with OS of diffuse large B-cell lymphoma (DLBCL). AG/GG genotype displayed an increased death risk (hazard ratio [HR] = 3.23; P < .001) compared with patients carrying the AA genotype [296].

The PMS1 gene is located on chromosome 2 (2q32.2), while PMS2 is found in clusters on chromosome 7 (7p22.1). Deficiency of PMS2 associated with cisplatin resistance in ovarian cancer patients [297].

1.3.4.2.3 Nucleoside excision repair

XPA is located on chromosome 9 (9q22.33) and it has 10 exons along with 4 transcripts. High XPA level predicts a poor prognosis in locally advanced nasopharyngeal carcinoma (NPC) patients treated with platinum-based chemo-radiotherapy [298]. Malignant ovarian cancer tissues from 13 patients, whose tumors were clinically resistant to therapy showed greater levels of total XPA mRNA and activity (P = 0.011) compared to clinically sensitive individuals to platinum therapy (n = 15) [208, 299, 300]. XPA expression in effusion tumor cells from metastatic ovarian carcinoma patients was shown associated with complete response to chemotherapy (P = 0.03): XPA expressed in greater than 25% of tumor cells from recurrent patients showed better PFS (P < 0.001) and OS (P = 0.04) [301, 302]. XPA rs1800975 (A23G), a promoter polymorphism, is associated with favorable prognosis of NSCLC receiving platinum based chemotherapy. XPA23 A/G+G/G genotypes were associated with significantly longer progression- free survival (PFS) (P = 0.001) and OS (P = 0.001) [303, 304]. Another important finding was, association between this rs1800975 and prognosis in 250

inoperable non-small cell lung cancer (NSCLC) patients treated with platinum-based chemotherapy. XPA 23 GA/AA genotypes were associated with poor survival (HR 1.55, p = 0.011 overall and HR 1.72, p = 0.008) and increased risk of death and progression (HR 1.73, p = 0.013 and HR 1.65, p = 0.016, respectively) [305]. Furthermore, rs1800975 was associated with relative risk (RR) of NSCLC patients receiving platinum based therapy. Homozygous G/G genotype showed a higher RR than patients with G/A or A/A genotype (74.1% vs 50.0%; OR: 0.09; 95% CI: 0.00–0.64; p = 0.013) [306]. rs1805160, XPA (Arg228Gln) was found significantly associated with recurrence from ovarian cancer in 125 Caucasian patients (HR (AA)= 12.06 (95% CI 1.22–118.76); HR(AG+GG) =9.11 (95% CI 1.12–73.90)) [307]. In addition, the XPA missense polymorphism rs104894131 (Cys108Phe) was associated with severe hematological toxicity in 55 NSCLC patients treated with platinum compound in combination with vinorelbine. [308].

XPC is located in chromosome 3 (3p25.1). It is 33,637 bp long and has 18 exons and 7 splice variants. XPC intronic variant rs2607755 was significantly associated with OS and disease free survival of platinum compound treated African Americans head and neck cancer patients (OS HR = 0.62 (0.45, 0.86), DFS HR = 0.51 (0.30, 0.86)) [309]. Epithelial ovarian cancer cohort study showed that median PFS of patients carrying the Lys/Lys and Lys/Gln+Gln/Gln genotype of the XPC mis-sense SNP rs2228001 (Lys939Gln/C2704A) polymorphism were 25 and 12 months (P=0.039), OS of patients was 31.1 and 27.8 (P=0.048). Additionally, patients with the Gln allele had an increased risk of death (HR=1.75; 95% CI=1.06-2.91) compared Lys/Lys genotype [310]. This polymorphism also showed similar association in NSCLC patients. The heterozygous A/C genotype carriers had a poorer response rate than the wild A/A genotype carriers (P = 0.023) [311]. Additionally, rs2228001 was associated with higher toxicity in NSCLC patients treated with platinum based therapy. T allele was associated with higher hematological toxicity (OR: 7.50, 95 % CI: 0.89-63.17, p = 0.036) [308]. rs2228001 CC genotype was also associated with ototoxicity in cisplatin-treated osteosarcoma patients (P-value= 0.042) [312]. Furthermore, rs2228001 (Lys939Gln) was significantly associated

with grade 3 or 4 neutropenia (p = 0.0026) and grade 3 or 4 hematological toxicity in blader cancer patients receiving platinum based chemotherapy. Gln/Gln or Lys/Gln + Gln/Gln genotypes of XPC had higher toxicity compared with Lys/Lys (OR: 10, p = 0.0070 vs OR: 6.3, p = 0.0069; respectively) [313]. rs3731108 (AG)/AA genotype versus the GG genotype was associated with prolonged a PFS of 21.3 months versus 13.4 months (P = .03) with stage III and IV papillary serous ovarian cancer. Similarly, rs1124303 (GT)/GG genotype versus the TT genotype was associated with a prolonged PFS of 22.8 months versus 14.9 months (P = .03) in same patient's cohort [314](Fleming ND.et al 2014 PMID: 21751198). Another SNP, XPC rs2228000 (Ala499Val) significantly associated with lung cancer survival (P = 0.002). Among 185 stages III-IV NSCLC patients treated with platinum-based chemotherapy [315].

XPB/ERCC3 is located at chromosome 2q14.3 with 15 exons and 9 splice variants. XPB/ERCC3 is also associated with platinum drug response in different cancers. For an example, rs3738948 was associated with platinum drug response in NSCLC patients. A/A genotype achieved poor response (68.3%) compare to A/G and G/G genotype (100% and 89.3%) (p = 0.008) [306].

XPD/ERCC2 (chromosome 19q13.32; 24 exons and 12 splice variants missense variant rs13181 (2251A>C or Lys751Gln) was associated with nephrotoxicty in platinum drug treated NSCLC patients [308]. G allele and T allele carrier patients showed significantly lower nephrotoxicity (p = 0.017 and p = 0.029) than in other patients [308]. The XPD synonymous SNP Asp312Asp rs1799793 (G312A), and XPD Lys751Gln (rs13181) were significantly associated with NSCLC patient's response to platinum drugs therapy [316-319]. Patients with Lys/Gln genotype was 0.400 times chemotherapy sensitivity compare with Lys/Lys genotype ($P < 0.05$). Furthermore, Lys/Lys vs Lys/Gln had significant differences in PFS. Whereas, Asp/Asp vs Asp/Asn genotype also showed similar association [316-319]. In 93 NSCLC patients significant difference was observed in OS between XPD Asp312Asp and Asp312Asn individuals (20.0 vs 12.4 months, P=0.04) [320]. Similar result was observed in another study in NSCLC patient's cohort, where median RFS (relapse-free survival

duration) CC, CT heterozygous and TT mutant allele genotypes were 28.3 months, 46.9 months and 80.1 respectively. Furthermore, median RFS was longer in mutant group (P = 0.018) [321]. Another study also reported that, XPD 312 C/T+T/T and XPD 711 C/T+T/T (rs1052555) associated with poor responses to platinum based chemotherapy in 496 and 375 NSCLC patient's cohort. Furthermore, median PFS and OS of patients of XPD 312 C/T+T/T genotype and XPD 711 C/T+T/T genotype was significantly lower compare to wild-type homozygous patients [322-324]. XPD Lys751Gln (A>C) polymorphism was also significantly associated with decreased PFS in Asians NSCLC patient's population treated with platinum based drug (P=0.879) [325]. Variant genotypes of XPD Asp(312)Asn, Asp(711)Asp and Lys(751)Gln formed haplotype and were significantly associated with poorer NSCLC survival. The most common haplotype GCA (Asp(312)Asn, Asp(711)Asp and Lys(751)Gln) exhibited significant risk effect on NSCLC survival (P = 0.001). ERCC2 Lys751Gln was also associated with survival outcome of metastatic breast cancer patients treated with carboplatin-containing chemotherapy [326]. These two SNPs were also associated with Grade 4 neutropenia and nephrotoxicity in advanced NSCLC, ovarian cancer and advanced gastric cancer (AGC) patients treated with platinum based chemotherapy [263, 265, 313, 327]. In 73 metastatic colorectal cancers patient cohort, rs13181 Lys/Lys genotype responded well (P = 0.015). The median survival for those with the Lys/Lys genotype of rs13181 was 17.4 compare to 12.8 and 3.3 months for patients with the Lys/Gln and Gln/Gln respectively (P = 0.002). Lys751Lys and the Asp312Asp polymorphism are linked and they form a haplotype (P < 0.001) [328, 329]. Similar result was observed in another study, ERCC2 rs13181T>G, the G allele was associated with reduced response and poor PFS and OS in Caucasians [330]. There is evidence that, these two polymorphism affected response and prognosis of bladder cancer patients treated with platinum based chemoradiotherapy [331]. XPD 751 A/A also showed a significant longer survival in the squamous cell carcinoma (P=0.034) and ovarian cancer treated with platinum based chemotherapy [249, 332]. These two polymorphisms were associated with event-free survival (EFS) in 130 patients with

high-grade osteosarcoma receiving platinum based therapy [333]. Similar result was reported from another study, where ERCC2 rs13181 G allele was significantly associated with shorter event-free survival (P-value=0.021)[312]. Colorectal cancer patients with XPD751 Lys/lys genotype were associated with 3.8-fold increase in failure of chemotherapy compared to Lys/Gln. The median time to progression (MTTP) were 11.3 months vs 2.9 months for patients with Lys/Gln and Gln/Gln genotypes ($P < 0.05$)[334]. Furthermore, XPD Lys751Gln rs13181 (A751C) was significantly associated with Event-free survival and OS in patients receiving oxaliplatin. Patients with C allele had a shorter median event-free survival and OS (6 months vs 11.6 months p = 0.008, 15.6 vs 25.3 months p=0.016) [335-337]. In addition, GA+AA genotypes of XPD rs1799793 were associated with improved response to platinum chemotherapy of gastric cancer patients and a significantly decreased risk of mortality [338]. AA genotype of ERCC2 rs1799793 was also associated with a better response to chemotherapy, longer OS in gastric cancer patients [339]). A synonymous SNP in XPD gene rs238406 Arg(156)Arg (homozygous minor allele) was associated with increased risk of grade 3 or 4 leukopenia (P=0.005) in non-small cell lung cancer patients in a Chinese population receiving platinum based chemotherapy. Further, Arg(156)Arg (rs238406) polymorphism was also significantly associated with grade 3 or 4 hematologic toxicity (; P = 0.009), and severe leukopenia (P= 0.005) [340, 341]. Furthermore, haplotype "CG" of (Arg(156)Arg-Asp(312)Asn) associated with grade 3 or 4 hematologic toxicity [340]. ERCC2/XPD rs238406 SNPs were poorer DFS and OS of 572 esophageal squamous cell cancer (ESCC) patient's treated with platinum drugs. Hazard ratio for TT vs. GG+GT for DFS and OS was HR = 1.43, P = 0.020 and HR = 1.52, P = 0.008 [342]. The XPD SNP rs50872 was associated with median survival duration, poor survival and grades 3 and 4 infections in NSCLC patient's population receiving platinum based treatment. Median survival for G/G, A/G, and A/A genotypes was 35.75, 36.07 and 16.75 months, respectively ($p<0.001$). Additionally, rs238405 and rs238416 was significantly related to OS [233].

XPF/ERCC4 is a 32,204 bases long gene located at chromosome 16p13.12. It has 14 exons along with 8 splice variants. Low XPF expression in HNSCC patients is associated with better response to induction chemo-radiotherapy, while high XPF expression correlates with a worse response (p=0.02) in advanced head and neck cancer receiving platinum therapy [343]. The XPF/ERCC4 (rs12926685) CT+CC genotype versus the TT genotype was associated with a prolonged PFS of 16.7 months versus 12.4 months (P = .03) in 139 patients with stage III and IV papillary serous ovarian cancer treated with platinum based therapy [314]. ERCC6-Q524* and ERCC4-A583T were related with favorable response to first-line platinum-based chemotherapy in advanced-stage high-grade serous EOC. ERCC4-A583T is missense mutation somatic mutation associated with heterozygous loss or complete loss of functional ERCC4, while ERCC6-Q524 is a nonsense mutation [344]. Synonymous SNP, rs1799801 in the ERCC4 gene significantly correlated with the relative risk (RR) in advanced NSCLC patients receiving platinum based therapy. Patients with the T/T genotype showed a significantly lower RR than compare to T/C or C/C genotype (25.0% vs 44.7%; OR: 3.30; 95% CI: 1.19–10.00; p = 0.021)[306].

ERCC5/XPG is located on chromosome 13 (13q33) with 15 exons and 8 splice variants. ERCC5 rs1799977 (His46His), a synonymous polymorphism, were associated with clinical outcomes of 378 advanced non-small cell lung cancer (NSCLC) patients receiving platinum-based chemotherapy regimens. NSCLC patients carrying the ERCC5 46T/T genotype had increased PFS and OS, with 0.52 (0.31-0.96) [345, 346]. In 228 advanced NSCLC patient's cohort among three tag-single nucleotide polymorphisms rs2094258, rs751402, and rs2296147 in the ERCC5 promoter region. The rs751402 AA genotype was associated with a better treatment response (AA vs. AG+GG: odds ratio (OR)=2.74, 95% confidence interval (CI) 1.04-7.26, P=0.036). Additionally, in the subgroup of patients with squamous cell carcinoma rs751402 AA genotype showed stronger association (AA vs. GG: OR=6.40, 95% CI 1.15-35.50, P=0.043; AA vs. AG+GG: OR=6.12, 95% CI 1.15-32.52, P=0.019). This result also suggested that ERCC5 rs751402 AA genotype increased

the chemotherapy response in advanced NSCLC, especially in patients with squamous cell carcinoma [347]. Furthermore, ERCC5/XPG rs2094258 and rs873601 also showed poorer DFS and OS among patients with resected esophageal squamous cell cancer (ESCC) patients. rs2094258, CT+TT vs. CC: for DFS hazards ratio = 1.68 and P = 0.012; and for OS hazard ratio = 1.99 and P = 0.0001), whereas for rs873601 GA+GG vs. AA, DFS hazard ratio = 1.59 and P = 0.024; OS hazard ratio = 1.91 and P = 0.0005 [342]. Another study reported similar associations where CT+TT genotype of rs2296147 had a significantly longer median PFS and OS than CC genotype. Similarly, rs2094258 AG+GG genotype had a longer median progression time and OS time than AA genotype ([348]. ERCC5 (D1104H) rs17655 was related with more infection (P=0.017) in 388 stage IIIB and IV NSCLC patients treated with platinum-based chemotherapy [349]. Additionally, ERCC5 (D1104H) rs17655 was associated with OS (P = 0.004) for lung cancer patients and with PFS for ovarian cancer receiving platinum-based chemotherapy [350, 351]. The polymorphism rs751402 located in the 5' UTR of ERCC5 generates a ORF (G= no ORF vs A= ORF) that controls the expression of ERCC5. AA expresses a significantly lower level of the uORF that results in higher levels of ERCC5 protein. cancer patients who carried the A allele were more resistance platinum chemotherapy due to relatively high rates of repair of platinum–DNA adducts within tumors [352]. Further, platinum drug treated NSCLC patients carrying the 5' UTR variant rs2296147 TT and the 3' UTR variant rs873601 GG genotypes were significantly associated with favorable outcome for PFS and OS [353]. Furthermore, a significant decreased risk of death was observed among patients carrying the rs2296147 TT genotype compared with the CC genotype [354]. In addition, patients with the rs1057768 (C/T) TT genotype had a significantly lower treatment response, short median PFS and OS compared with the rs1057768 CC genotype respectively in NSCLC patient receiving platinum based therapy [354].

The ERCC1 gene is located on chromosome 19 (19q13.32). It is 71,496 bases long gene along with 14 exons and 17 transcripts or splice variants. Increased ERCC1 expression associated with

Platinum drugs resistance in various cancers including ovarian, NSCLC, nasopharyngeal, esophageal, cervical, head and neck squamous carcinoma, liver, osteosarcoma, lung adenocarcinoma, biliary tract adenocarcinoma, mesothelioma, pulmonary adenocarcinoma, bladder cancer, gastric cancer, adrenocortical carcinoma and urothelial carcinoma [208, 211, 213, 267, 299, 302, 355-373]. EOC patients treated with platinum-based chemotherapy were significantly associated with time to progression (TTP) and OS. OS was significantly longer with low expression group compared with patients showing high expression of ERCC1 protein [374]. Another study reported that patients with ERCC1-positive tumors had significantly shortened progression-free ($p<0.00001$) and OS ($p=0.0006$) compared to patients with ERCC1-negative tumors [375-378]. In advanced cervical cancer patients with negative ERCC1 expression associated with response to Platinum based chemotherapy ($p=0.021$) as well as disease-free survival for this Patients ($p=0.046$) [379]. ERCC1 expression also found to be associated with outcome in gastric cancer patients receiving platinum drugs. Meta-analysis of twenty-one studies involving 1,628 patients of gastric cancer showed high ERCC1 expression was significantly associated with shorter OS and lower response to chemotherapy in advanced GC patients receiving platinum based chemotherapy (HR 1.83; 95 % CI 1.45-2.31; $P < 0.001$; RR 0.49; 95 % CI 0.38-0.62; $P < 0.001$) [380-388]. Many studies have demonstrated a correlation between low levels of ERCC1 and increased survival in NSCLC patients treated with cisplatin [389] as well as carboplatin [390-416]. On the other hand, high levels of expression of ERCC1 mRNA and protein both found to be associated with resistance to cisplatin in NSCLC [389]. Non-small cell lung cancer (NSCLC) and gastric cancer patients treated with platinum-based chemotherapy showed absence or low expression of ERCC1 was associated with longer disease free survival DFS ($P=0.037$, $P < 0.0001$, $P = 0.018$) and longer OS ($P = 0.007$) [417]. Low ERCC1 expression level was correlated with longer OS (HR (95% CI) of NSCLC patients treated with platinum based therapy. Additionally, NSCLC patients with ERCC1 low expression also had a better PFS ($P < 0.05$) for platinum based chemotherapy. On the other hand, high expression of

ERCC1 was found to be associated with poor prognosis (P<0.001) and decreased survival rates in lung cancer patients treated with platinum based chemotherapy [417].

A multivariate analysis in platinum-based chemotherapy, low expression of ERCC1 was shown to be an independent predictive factor for response to chemotherapy (P = 0.018), time to progression (P = 0.025), and OS (P = 0.038) [418]. As ERCC1 increased, the probability of response decreased (P = 0.083), the risk of progression (ERCC1, HR = 1.59, P = 0.002; and death (ERCC1, HR = 1.62, P = 0.008) increased [418]. Tumor samples from patients enrolled in the International Adjuvant Lung Cancer Trial concluded that patients with ERCC1-negative tumors appear to benefit from adjuvant cisplatin-based chemotherapy [419-422]. Similarly, for patients treated with carboplatin, ERCC1 expression was correlated with OS. The median OS for high expressed ERCC1 patient's vs low expressed group was 9.5 months (95% CI 6.7 to 11.8) vs 15.6 months (95% CI 11.6 to 24.8) [423]. In another study, a NSCLC patient's group treated with carboplatin and paclitaxel combination therapy, the prognosis was significantly better of the ERCC1-negative group compare to ERCC1-positive group (P= 0.014) [387, 424, 425]. Additionally, meta-analysis evaluated the relationship between ERCC1 expressions and the clinical outcomes including overall response rate (ORR), OS or PFS on 1129 patients with small cell lung cancer (SCLC) receiving platinum-based chemotherapy. Analysis indicated that positive/high ERCC1 expression was associated with unfavorable OS (HR=1.18, 95%CI=1.02-1.37) and PFS (HR=1.46, 95%CI=1.14-1.88) [426-428]. ERCC1 was also a predictor of shorter survival in Asians and Caucasians for head and neck squamous cell carcinoma (HNSCC) patients treated with platinum-based treatment. High ERCC1 expression was associated with unfavorable OS (p = 0.009), PFS (p = 0.000) and ORR (p = 0.044) [429, 430]. Furthermore, ERCC1 was also associated with higher objective response, median PFS, and median OS in patients with advanced bladder cancer treated with platinum-based chemotherapy. Patients with ERCC1 low/negative expression showed significantly prolonged median OS time and the median PFS time compared to ERCC1 high/positive expression (hazard ratio 0.69, 95% CI 0.54-0.89, P=0.004, and

hazard ratio 0.76, 95% CI 0.66-0.89, P=0.000, respectively) [384, 431, 432]. In malignant pleural mesothelioma (MPM) patients receiving platinum based chemotherapy, a significant association was found between ERCC1 protein levels and OS (P = .032) [292, 433]. ERCC1 was also associated with clinical outcomes in patients with pulmonary adenocarcinoma treated with pemetrexed/cisplatin as first-line chemotherapy. Low ERCC1 expression was significantly associated with better relative risk (RR) (p = 0.015) and longer PFS (p = 0.004). Low ERCC1 expression was also associated with longer OS (p = 0.003) [434]. Nasopharyngeal cancer patients treated with platinum (cisplatin) based chemotherapy also showed that ERCC1-negative tumors had longer disease-free survival (p = .076) and OS (p = .013) than patients with ERCC1-positive tumors [435]. Similarly, in biliary tract adenocarcinoma (ABTA) patients treated with platinum-based chemotherapy median PFS was (4.6 vs. 1.9 months, p = 0.014) and OS was (9.1 vs. 7.9 months, p = 0.017) in ERCC1-negative group than in ERCC1-positive group, respectively. Furthermore, disease control rate (DCR) was also better in patients with ERCC1 negative than in ERCC1 positive patients (p = 0.048) [415]. ERCC1 was also found to associated with pancreatic cancer patients treated by platinum based chemotherapy Median survival, longer time to progression was significantly higher in group with low ERCC1 levels (Median survival, 11.9 versus 9.9 months, $p \leq 0.05$) [436]. Similarly, ERCC1 expression also correlated with lack of response (P=0.006) and poor disease-specific (P=0.020) and OS (P=0.040) in gastro-esophageal cancer patients receiving platinum-based chemotherapy [437]. Furthermore, ERCC1 expression was strongly correlated with poor prognosis and OS in adrenocortical carcinoma (ACC) patients treated with platinum-based chemotherapy. OS was eight months in high ERCC1expressed group whereas, 24 months in low ERCC1 expressed group (P=0.004) [438, 439].

Figure 1.11 summarizes the structure of ERCC1 gene along with pharmacogenomically relevant polymorphisms that are associated with treatment outcome of platinum based chemotherapy. ERCC1 rs3212986 (C8092A), a missense mutation (Gln506Lys), was found to be associated with

response to platinum based therapy in gastric cancer patients. AA genotype was associated with lower rates of complete remission and partial remission along with higher risk of death in gastric cancer patients [440]. Similarly, rs3212986, CA and AA genotypes associated with a poorer response to chemotherapy compared to the CC genotype (P = 0.04, P = 0.01). Patients with the AA genotype associated with longer OS time when compared with the CC genotype (34.91 months vs. 51.19 months, P = 0.003) in gastric cancer patients receiving platinum based therapy [441, 442]. In gastric cancer patients another SNP rs11615 was also found with clinical outcome. TC+CC genotypes were associated with significantly decreased risk of mortality (hazard ratios = 1.71, 95% CI, 1.06-2.72) [338]. CC genotype of ERCC1 rs11615 was associated with a better response to chemotherapy in gastric cancer patients. CC genotype significantly associated with a longer OS of gastric cancer [330, 339]. rs3212986 was also associated with PFS in NSCLC patients. ERCC1 mRNA levels were lower in CC homozygous patients compared with the patients carrying the A allele (p = .024)[443]. NSCLC patients with rs3212986 CA genotype significantly contributed to poorer disease-free survival (DFS) and OS than those with the CC genotype (p=0.037 and 0.004) [444, 445]. rs3212986 was also found to be associated with a significantly increased risk of grade 3 or 4 gastrointestinal toxicity (P = 0.03) in NSCLC patients. Patients with the variant allele of the SNP showed grade 3 or 4 gastrointestinal toxicity (odds ratio, 2.33; 95% confidence interval, 1.07-5.05) [417]. Among 128 advanced NSCLC patients treated with platinum-based chemotherapy, a statistically significant association was observed between the rs3212986 (C8092A) polymorphism and OS (P = 0.006, by log-rank test), with median survival times of 22.3 (C/C) and 13.4 (C/A or A/A) months, respectively, suggesting that any copies of the A allele were associated with poor outcome [444, 445]. Furthermore, rs3212986 was found to be associated with survival of platinum chemotherapy treated Esophageal squamous cell carcinoma patients (P=0.01). Homozygous mutant allele (AA) showing the most significantly reduced survival (P=0.04) compared to (CC) [446]. ERCC1 polymorphism rs3212986 C/A was also associated with PFS and OS of epithelial ovarian cancer (EOC) patients.

The CA or AA genotypes could influence OS (HR = 1.28, 95% CI = 1.05-1.56; and HR = 1.55, 95% CI = 1.17∼2.05, respectively) [247, 447-449]. On the other hand, rs3212986, C/C wild-type genotype significantly associated with PFS (P = 0.034) in platinum treated malignant mesothelioma (MM) patients [450].

In NSCLC, the synonymous coding SNP rs11615 (Asn118Asn), was also found to be associated with response against platinum based chemotherapy. Patients with C/C showed better response compare to C/T + T/T genotype (P = 0.007) [243, 257, 315, 451]. Similarly, OS was significantly longer in patient's C/T or T/T allele compare to C/C allele (P=0.014) for rs11615 [332, 426, 452]. rs11615 was also found to be associated with ERCC1 expression and platinum drug treated EOC patient's outcome. TT genotype had a significantly poor response (OR = 6.22, 95%CI:1.12-34.42), shorter PFS (HR = 2.19, 95%CI:1.14-4.22, P = 0.009) and OS (HR = 2.22, 95%CI:1.06-4.64, P = 0.021) to platinum-based chemotherapy [366, 382, 453, 454]. On the other hand, rs11615 was also associated with the risk of anemia in platinum drug treated ovarian cancer patient. Risk was higher for T allele compare to C allele (T > C; p = 0.031, OR = 1.61, 95% CI = 1.04-2.50) [249, 455, 456]. In osteosarcoma patient's rs11615, T/T was strongly associated with a higher event free survival compared to C/C genotype [457]. Furthermore, ERCC1 C/C significantly associated with better survival and longer survival of colorectal cancer patients treated with platinum based chemotherapy [273, 328, 458, 459]. Furthermore, rs11615 polymorphism was associated with therapeutic response in Caucasian patients. Meta-analysis study reported that C allele was found to be associated with better patient response platinum based therapy [460]. Furthermore, rs11615 TT genotype and T allele and rs3212986 AA and A allele were associated with a poor response and higher risk of death from platinum treated NSCLC patients [255, 454, 455, 461-471]. In a study concerning ovarian cancer patients (n=280) median OS was 45, 40 or 30 months for the rs3212986 (C8092A) genotype CC, CA and AA, respectively. Patients with CA+AA versus CC in had increased risk of death (p=0.077) [265, 327]. rs3212986, rs11615 were significantly associated with OS in T4 breast cancer patients treated with

chemotherapy containing platinum (p-values = 0.036 and 0.004, respectively). combination of these two genotypes found to be played a role as significant prognostic factor in T4 breast cancer patients receiving platinum-based chemotherapy (p-values = 0.022 and 0.049, respectively) [472]. rs11615, the T/T genotype was found to be associated with prolonged median cancer-specific survival (p = 0.026) in platinum treated advanced urothelial cancer (UC) [473]. Additionally, these two SNPs rs11615 and rs3212986 were associated with higher risk of nephrotoxicity in ovarian cancer and neurotoxicity in advanced gastric cancer patients [456]. Another 2 SNPs in ERCC1 5'-flanking region, -433T>C and 262G>T was associated with decreased ERCC1 RNA expression. 262G allele is associated with better drug response and longer survival time compared with the 262T allele in SCLC patients [474].

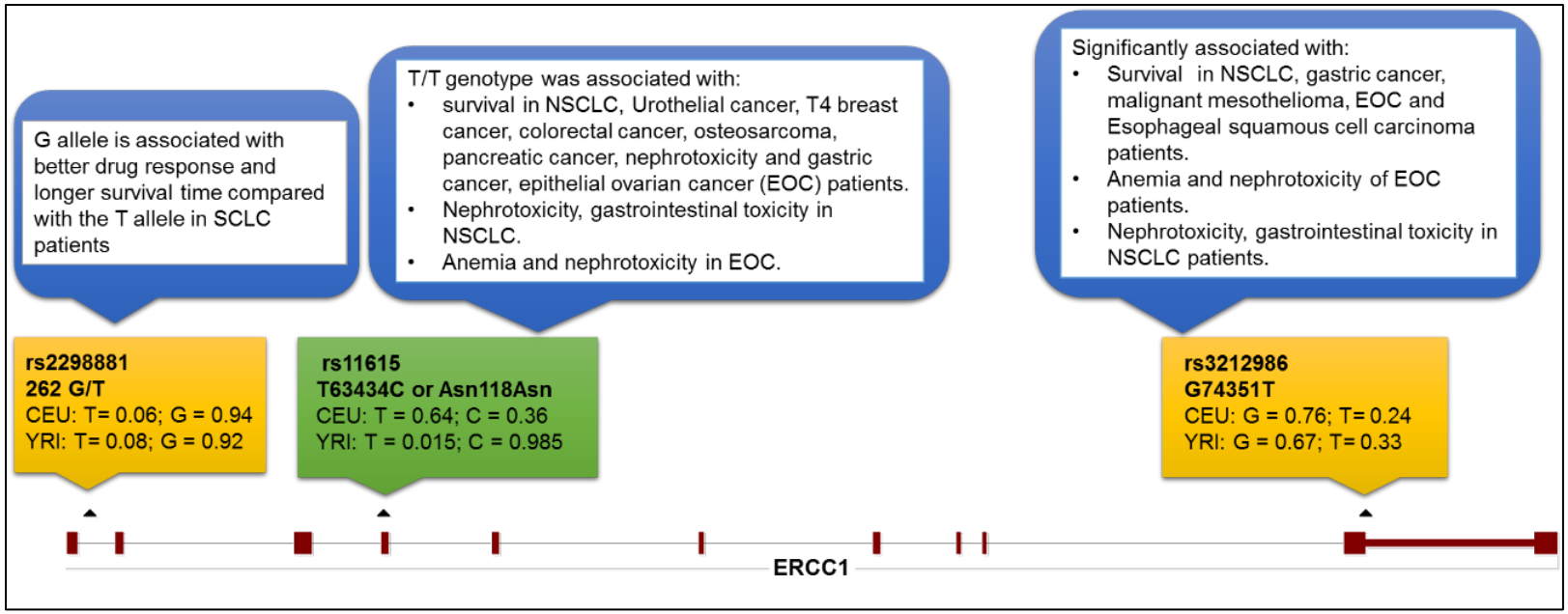


Figure 1.11 Genetic variants in ERCC1 associated with survival/outcome and/or toxicity in patients treated with platinum drug-based chemotherapy (Green box denotes coding SNP; Yellow boxes represent non-coding SNPs).

Table 1.5 Summary of SNPs in NER pathway genes associated with Platinum chemotherapy.

SNP ID	Gene symbol	Cancer Type	Associated Phenotype	Associated Genotype	PubMed ID (s)
rs1800975	XPA	NSCLC	PFS, OS	XPA23 (A/G+G/G) were associated with significantly longer PFS (P = 0.001) and overall survival (OS) (P = 0.001)	19430706
			Survival, Progression, Risk	PA23 (A/G+G/G) was associated with poor survival (p = 0.008) and increased risk of death(p = 0.013), progression (p = 0.016) and relative risk (RR). Homozygous G/G genotype showed a higher RR than patients with G/A or A/A genotype (p = 0.013).	22511383, 25069034
rs104894131	XPA	NSCLC	Toxicity	XPA -2166T>C was associated with severe hematological toxicity in 55 NSCLC patients	26193985, 23617284
rs1805160	XPA	Ovarian cancer	Recurrence	HR (AA)= 12.06 (95% CI 1.22–118.76); HR(AG+GG) =9.11 (95% CI 1.12–73.90) in 125 Caucasian patients	17825393
rs2607755	XPC	Head and Neck cancer	OS, DFS	rs2607755 vs OS (HR = 0.62 (0.45, 0.86)); vs DFS (HR = 0.51 (0.30, 0.86)) in African Americans head and neck cancer patients	24487794
rs2228001	XPC	Ovarian cancer	OS	rs2228001 (C/A) vs OS (P=0.048)	23621265
		NSCLC	Response, Toxicity	Heterozygous A/C genotype carriers had a poorer response rate and higher hematological toxicity (p = 0.036.) than the wild A/A genotype	19434073, 26193985
		Osteosarcoma	Toxicity	rs2228001 CC genotype was associated with ototoxicity (P-value= 0.042)	19434073
		Bladder cancer	Toxicity	rs2228001 was significantly associated with grade 3 or 4 neutropenia (p = 0.0026) and grade 3 or 4 hematological toxicity.	21047201
rs1124303	XPC	Bladder cancer	PFS	rs1124303 (GT/GG) genotype (PFS= 22.8 months) vs TT genotype (PFS= 14.9	21751198

				months) was associated with a prolonged PFS(P = .03)	
rs3731108	XPC	Ovarian cancer	PFS	rs3731108 (AG/AA) genotype (PFS=21.3 months) vs GG genotype (PFS=13.4 months) was associated with prolonged progression-free survival (PFS) (P = .03) in patients with stage III and IV papillary serous ovarian cancer.	21751198
rs2228000	XPC	NSCLC	Survival	rs2228000 vs survival (P = 0.002) among 185 stages III-IV NSCLC patients.	21739480
rs3738948	XPB/ERCC3	NSCLC	Response	rs3738948 A/A genotype achieved poor response (68.3%) compare to A/G and G/G genotype (100% and 89.3%) (p = 0.008)	25069034
rs13181	XPD/ERCC2	NSCLC	PFS, RFS, Toxicity	Patients with rs13181 Lys/Gln genotype was 0.400 times chemotherapy sensitivity compare with Lys/Lys genotype (P < 0.05). Lys/Lys vs Lys/Gln had significant differences in PFS and nephrotoxicity (p = 0.017)	26193985, 26124006, 15173214, 16351803, 23977265, 25596702, 24260311
		Colorectal cancer	Response, EFS, OS	Patients cohort with rs13181 Lys/Lys genotype responded well (P = 0.015). Median survival: Lys/Lys =17.4 months; Lys/Gln=12.8 months; Gln/Gln=3.3 (P = 0.002)in 73 metastatic colorectal cancers patient's cohort . rs13181 was significantly associated with EFS and OS. Patients with rs13181 C allele had a shorter median EFS and OS in patients receiving oxaliplatin.	11751380, 26125734, 21278243, 18085999, 18797464, 19432884, 22967467
		Gastric cancer	Respose, PFS, OS	rs13181T>G, the G allele was associated with reduced response and poor PFS and OS in Caucasians gastric and colorectal cancer patients.	21278243
		squamous cell carcinoma,		rs13181 showed a significant longer survival in the squamous cell carcinoma (P=0.034) and ovarian cancer.	21676483, 25881102

		Ovarian cancer			
rs1799793	XPD/ERCC2	Gastric cancer	Response, OS, Risk	rs1799793 was associated with improved response, longer overall survival (OS) and a significantly decreased risk of mortality.	26622786, 25542228
		NSCLC	OS	Significant difference was observed in overall survival (OS) between XPD rs1799793 Asp312Asp and XPD Asp312Asn individuals (P=0.04)	26124006, 15173214, 16351803, 23977265, 24841663, 23534713, 22374424, 24446315
rs13181, rs1799793	XPD/ERCC2	NSCLC, Ovarian cancer and gastric cancer toxicity	Toxicity	rs13181, rs1799793 were associated with Grade 4 neutropenia and nephrotoxicity .	16649224, 19786980, 19332728 , 21047201
		Bladder cancer	Response, Prognosos	rs13181, rs1799793 affected response and prognosis.	16880786
		Osteosarcoma	EFS	rs13181, rs1799793 were associated with event-free survival (EFS) in 130 patients with high-grade osteosarcoma receiving platinum based therapy	21826087, 19434073
rs150461093	XPD/ERCC2	NSCLC	Toxicity	rs150461093 associated with nephrotoxicty.	26193985
rs1052555	XPD/ERCC2	NSCLC	Response, PFS, OS	rs1052555 XPD 711 C/T+T/T was associated with poor responses in 496 and 375 NSCLC patient's cohort. Median PFS and OS of patients of XPD 711 C/T+T/T genotype was significantly lower compare to wild-type homozygous patients.	23534713, 22374424, 24446315
rs238406	XPD/ERCC2	NSCLC	Toxicity	rs238406 vs grade 3 or 4 hematologic toxicity (P = 0.009); vs severe leukopenia (P= 0.005)	22479369, 19458053, 19458053
		Squamous cell cancer	OS, DFS	rs238406 SNPs was associated with poorer DFS and OS.	27246611
rs50871	XPD/ERCC2	Head and Neck cancer	OS, DFS	rs50871 vs OS (HR = 0.80); vs DFS (HR = 0.67).	24487794

rs50872, rs238405 , rs238416	XPD/ERCC2	NSCLC	Survival, Toxicity	Median survival for rs50872 G/G, A/G, and A/A genotypes was 35.75, 36.07 and 16.75 months, respectively (p<0.001); rs50872 was also associated grades 3 and 4 infections; rs238405 and rs238416 were significantly related to OS in NSCLC patients receiving platinum based treatment.	22608006
rs12926685	XPF/ERCC4	Ovarian cancer	PFS	rs12926685 CT/CC genotypes (PFS=16.7) vs TT genotype (PFS= 12.4 months)(P = .03)	21751198
rs3136038, rs3136130	XPF/ERCC4	Head and Neck cancer	Survival	rs3136038 and rs3136130 were associated with survival.	24487794
rs1799801	XPF/ERCC4	NSCLC	Risk	Patients with the rs1799801 T/T genotype showed a significantly lower RR than compare to T/C or C/C genotype (p = 0.021.)	25069034
rs751402	ERCC5/XPG	Squamous cell cancer	Response	rs751402 AA genotype was associated with a better treatment response [AA vs. AG+GG: OR=2.74, 95% CI=1.04-7.26, P=0.036)	23211354
		Pediatric ependymoma	Resistance	rs751402 AA patients were more resistance platinum chemotherapy due to relatively high rates of repair of platinum–DNA adducts within tumors. .	26338418
rs2296147	ERCC5/XPG	NSCLC	PFS, OS	rs2296147 CT+TT genotype had a significantly longer median PFS and OS than CC genotype.	23621222, 24615519, 23621222
rs2094258, rs873601	ERCC5/XPG	Esophageal cancer	OS, DFS	rs2094258 CT+TT vs. CC: for DFS (HR = 1.68 and P = 0.012); for OS (HR = 1.99 and P = 0.0001). rs873601 GA+GG vs. AA, DFS (HR = 1.59 and P = 0.024); OS (HR= 1.91 and P = 0.0005).	27246611
rs2094258	ERCC5/XPG	NSCLC	Progression, Survival	rs2094258 AG+GG genotype had a longer median progression time and OS time than AA genotype.	24353624

rs873601	ERCC5/XPG	NSCLC	Outcome	rs873601 GG genotype was significantly associated with favorable outcome for PFS and OS.	24615519
rs17655	ERCC5/XPG	NSCLC	Infection	rs17655 was related with more infection (P = 0.017).	23118991
		lung cancer patients, Ovarian cancer	PFS, OS	rs17655 was associated with OS (P = 0.004) for lung cancer patients and PFS for ovarian cancer.	17855454, 22158331
		Bladder cancer	Toxicity	rs17655 is associated with grade 3 or 4 neutropenia.	21047201
rs1057768	ERCC5/XPG	NSCLC	Response	rs1057768 TT genotype had a significantly lower treatment response, short median PFS and OS compared with the CC genotype.	23621222
rs11615	ERCC1	Urothelial cancer	Survival	rs11615, the T/T genotype was found to be associated with prolonged median cancer-specific survival (p = 0.026).	23148636
		Pancreatic cancer	Survival	rs11615 was associated with survival.	24403499, 18425336
		Osteosarcoma	EFS	rs11615, T/T was strongly associated with a higher event free survival compared to C/C genotype	23098477
		Colorectal cancer	Survival	rs11615, significantly associated with longer survival	22994779, 22567180, 16144907
rs3212986	ERCC1	Malignant Mesothelioma	PFS	rs3212986, C/C wild-type genotype significantly associated with progression-free survival (PFS).	21765044
		Esophageal cancer	Survival	rs3212986 was found to be associated with survival.	25191856
rs3212986, rs11615	ERCC1	Gastric cancer	Remission, OS	rs3212986 and rs11615, were associated with remission, risk of mortality and OS.	27173253, 26823845, 26722542, 26622786, 25542228, 21278243

		NSCLC	PFS, DFS, OS, Toxicity	rs3212986 and rs11615 were associated with PFS, DFS, OS, death risk, Nephrotoxicity, gastrointestinal toxicity.	25647444, 19361884, 25250341, 24958519, 22532140, 21739480, 22932088, 24859833, 23727606, 22031394, 23226053, 20462983, 20351547, 20143185, 20070981, 19362955, 24370899, 24338713, 24045016, 21676483, 15277258, 15297394, 15746057, 17222938
		Ovarian cancer	PFS, OS, Toxicity	rs3212986 and rs11615 polymorphism in ERCC1 were associated with PFS, OS , anemia and nephrotoxicity in epithelial ovarian cancer (EOC)	24499239, 23632208, 19203783, 18640939, 24444563, 25881102, 22329723, 21496891, 19786980, 19332728, 18024864, 17961161, 23632208
		Brest cancer	OS	rs3212986, rs11615 were significantly associated with OS in T4 breast cancer	25253066
rs2298881	ERCC1	SCLC	Response, Survival	262G allele is associated with better drug response and longer survival time compared with the 262T allele	18451256

1.3.4.2.4 Homologous recombination DNA repair (Double strand break) gene

The XRCC1 gene is located on chromosome 19q13.31 (17 exons, 9 splice variants). XRCC1 expression is a prognostic marker for survival in different cancer patients receiving platinum therapy. Positive XRCC1 was associated with twofold increase risk of death ($p = 0.007$) and progression ($p < 0.0001$) and progression free survival [$p = 0.003$], as well as adverse reactions in ovarian cancer [217]. XRCC1 expression was associated with disease-specific mortality (P=0.024) in bladder cancer patients treated with platinum chemotherapy [359].

The major polymorphisms in this gene associated with platinum based chemotherapy are the missense SNPs rs1799782 (Arg194Trp/C580T) and rs25487 (Arg399Gln/G1196A).

The rs1799782 TT genotype and the CT genotype showed higher efficacy the CC genotype whereas, rs25487 GG genotype was found more sensitive to chemotherapy compared with the AG genotype [256, 289, 317, 324, 346, 397, 468, 475-482].

rs25487 399A/A genotype was significantly associated with longer PFS and OS in 378 lung cancer patients treated with platinum based chemotherapy[260, 345, 483, 484]. XRCC1 rs25487 is also associated with the clinical outcome of platinum-based chemotherapy in gastric cancer patients. A allele was found to be significantly associated with poor OS (HR = 1.40; 95%CI = 1.04-1.90) of gastric cancer for platinum-based chemotherapy[327, 485, 486]. XRCC1 rs25487 was also associated with reduced relative risk (RR) to platinum-based chemotherapy in gastric and colorectal cancer patients. Odds ratio for A/G + A/A vs G/G was 0.73; 95% CI, 0.55-0.96 [487-489]. Severe neutropenia was associated with rs25487 Arg/Arg genotypes in ovarian cancer patients [265]. rs25487 was also shown significantly associated with improved cancer-specific survival in bladder cancer patients receiving platinum drugs (P=0.009) [331]. Ovarian cancer patients bearing the Trp/Trp genotypes of the missense mutation rs1799782 had longer survival time than Arg/Arg genotype and rs25487, Gln/Gln genotypes had 0.44-fold risk of death than Arg/Arg genotype. The combination of both

194 Trp and rs25487 (Arg399Gln) 399 Gln allele was found to be associated with decrease risk of death in ovarian cancer patients treated with platinum based drugs [490, 491].

Furthermore, rs1799782 was also associated with severe hematological toxicity. The odds ratio (OR) of was significantly lower in patients carries T allele (OR = 0.22, 95 % CI: 0.06-0.82, p = 0.018) in NSCLC patients treated by platinum based drugs [308].

In addition, rs1799782 (Trp vs. Arg, OR=1.342, 95% CI: 1.176) was shown associated with increased risk of cervical cancer and better response to platinum drugs, whereas rs25487 allele A was linked with a poor response [492-495]. rs25487 was also found to be associated with clinical response in platinum drugs treated NSCLC patients. GA or GG genotypes had better response than AA genotype P = 0.028). Additionally, Patients carrying CT or TT genotypes of rs1799782 of XRCC1 showed more sensitive to platinum-based chemotherapy compared to patients with CC genotype (P = 0.002) in NSCLC patients [496]. A meta-analysis study that included total of 53 studies with 7433 advanced lung cancer patients reported that Trp/Trp and Trp/Arg genotypes of rs1799782 was found to be associated to better response rates to platinum-based chemotherapy compared to Arg/Arg genotype (TrpTrp+TrpArg vs. ArgArg: odds ratio (OR) = 2.02, 95% CI, 1.66-2.45) whereas the Gln/Gln and Gln/Arg genotypes of rs25487 were significantly associated with the poorer response rates compared with the Arg/Arg genotype (GlnGln +GlnArg vs. ArgArg: OR = 0.68, 95% CI, 0.54-0.86)[497]. In another meta-analysis study also concluded that rs1799782 (Arg194Trp) was significantly associated with the efficacy of platinum-based chemotherapy in NSCLC [498](Fan X. et al PMID: 26458583). In 122 pancreatic cancer stage-III/IV patients' rs25487 and rs1799782 were associated with clinical response to platinum drug therapy. Arg399Gln + Gln399Gln genotypes had a worse prognosis in pancreatic cancer patients receiving platinum based therapy [499].

XRCC2 is a 31,387 bases long gene located at chromosome 7q36.1. It has 3 exons and 2 splice variants. A 5′ flanking SNP rs6464268 in XRCC2 was associated with toxicity in esophageal adenocarcinoma patients, who received cisplatin-based preoperative radio-chemotherapy. Patients with AA genotype had grade 3–4 radiation-related toxicity, compared to GG or GA genotypes (P = 0.005) [275]. Further, the XRCC2 41657T allele of the polymorphism C41657T was associated with significantly higher carboplatin and cisplatin sensitivity than lung cancer patients with 41657C/C genotype (P = 0.046 and P = 0.039, respectively) [500]

XRCC3 variants (Chromosomal location 14q32.33; 10 exons; 18 Splice variants; length=17,897 bases) were found to contribute patient outcome in various types of cancers undergoing platinum chemotherapy. The missense coding SNP rs861539 (C18067T/Thr241Met) was found associated with clinical outcome of 1024 NSCLC patient's cohort receiving platinum based therapy. CC genotype was related to more resistance to platinum-based chemotherapy when compared patients with CT or TT genotypes (P = 0.009) [501]. Another meta-analysis study also reported that rs861539 was associated with good response to platinum-based chemotherapy in NSCLC patients. Odds ratio (OR) was 1.509 at 95% CI: 1.099-2.072 for ThrMet/MetMet vs. ThrThr. [502]. Similar kind of association was observed in platinum drug treated ovarian cancer patients, where Thr/ Thr genotype associated with increased risk of death compare to Met/Met genotype [491]. Another study showed that XRCC3 241 Thr/Met or Met/Met allele was associated with longer OS than Thr/Thr allele (19.0 m vs. 12.5 m, p=0.081) in NSCLC patients treated with platinum based combination therapy [322, 503]. The XRCC5 polymorphisms rs1051685 and rs6941 were associated with hematologic toxicity in lung cancer patient treated with platinum based compound [501].

1.3.4.2.5 Translesional Replication DNA repair (SOS) genes

REV1, REV3, and REV7 are involved in translesional DNA repair mechanism in platinum drug metabolism pathway. The G/G genotype of rs240966 (as intronic variant) and A allele of rs456865

(an intronic variant) were associated with higher grade 3 or 4 gastrointestinal toxicity in patients [504].

NSCLC patients (n=663) treated with platinum based therapy reported that rs240969 and rs3218573 was significantly associated with the treatment response (P=0.0082 and 0.036, respectively). Additionally, patients with G/G genotype of rs240966 or A allele of rs456865were found to be associated with grade 3 or 4 gastrointestinal toxicity [504].

POLB is located in chromosome 8 (8p11.21). It has 16 exons with 17 splice variants. High POLB expression had significantly poorer prognosis than patients with low expression ($P < 0.05$) in 97 colorectal cancer patients (CRC) ([505]. The non-synonymous polymorphism in POLB, P242R, was associated with OS ($P = 0.018$) in 456 lung cancer patients treated with platinum-based agents [350]. is located in chromosome 5 (5q13.3). Finally, rs3213801 within the gene Polymerase (DNA Directed) Kappa (POLK; chromosome 5q13.3) was found to be associated with increased risk of death in NSCLC patients treated with Platinum-Based Chemotherapy. Patients with AA genotype had a shorter survival compared with AG or GG genotype (P= 0.010) [506].

Table 1.6 Summary of SNPs in MMR, NHEJ and SOS pathway genes associated with Platinum drug chemotherapy outcome.

SNP ID	Gene symbol	Gene Function	Cancer Type	Associated Phenotype	Associated Genotype	PubMed ID (s)
rs1412125, rs2249825	HMGB1	pt-DNA adduct recognition	Lung Cancer	Response	rs1412125 and rs2249825 were significantly associated with response in Chinese lung cancer patients.	24684392
rs1805412	PARP1		NSCLC	PFS	rs1805412 (Va1762Ala) -762 CC genotype was significantly associated with poor PFS (CC vs. CT/TT: adjusted HR = 1.90, 95 % CI = 1.02-3.52)	23479135
hMSH2 gIVS12-6T/C	MSH2	Mismatch repair pathway	NSCLC	Response	hMSH2 gIVS12-6T/C associated with response to cisplatin- or carboplatin-based chemotherapy in peripheral lymphocytes from advanced NSCLC patients belonging to the Chinese population.	20458443
rs26279, rs1105524	MSH3		NSCLC	PFS	rs26279 (Ala1054Thr) was associated with sensitivity to platinum-based chemotherapy (P = 0.014). rs1105524 G/A and A/A genotypes showed shorter PFS than patients with the G/G genotype (P = 0.04).	25966119
rs1799977	MLH1		B-cell lymphoma	OS, risk	rs1799977 was associated with OS. AG/GG genotype displayed an increased death risk (hazard ratio [HR] = 3.23; P < .001) compared with patients carrying the AA genotype.	21156845
rs5742933	PMS1		NSCLC	OS	rs5742933 was found significantly associated with poorer prognosis on OS in a case-cohort of 568 NSCLC patients	21739480

rs1799782	XRCC1	Double strand break repair pathway	Ovarian cancer	Risk	rs1799782 associated with decrease risk of death	22983827, 22938418
			Mesothelioma	OS	rs1799782 was associated with overall survival(OS)	22982660
rs25487	XRCC1		Bladder cancer	Survival	rs25487 significantly associated with improved cancer-specific survival	24649183
			Ovarian cancer	Toxicity	rs25487polymorphism was associated with neutropenia	19786980
				OS, risk	rs25487 (Arg399Gln/G1196A) was associated with the OS and risk. A allele was found to be significantly associated with poor OS.	24634242, 24465544, 19332728, 24224851, 17593927, 11712813
			Esophageal cancer	Response, Survival	rs25487 was found to be associated with the response and poor survival	16785472
rs25487 and rs1799782	XRCC1		NSCLC	Response, Toxicity	rs25487 and rs1799782 were associated with clinical response and hematological toxicity.	25784983, 24938464, 24737519, 24729390, 24446315, 23549037, 23479135, 23167352, 22705987, 22551904, 22339849, 20719167, 19362955, 19157633, 15173214, 27248474, 26585370, 26458583, 26193985, 24782167, 21805378, 17504986, 22009704

			Cervical cancer	Risk, Response	rs1799782 was associated increased risk of cervical cancer and better response to platinum drugs. rs25487 A allele was linked with a poor response	23464469, 19563645, 18851872, 16875718
			Pancreatic cancer	Response	rs25487 and rs1799782 was associated with clinical response	22026922
rs6464268	XRCC2		Esophageal cancer	Toxicity	Patients rs6464268 AA genotype had grade 3–4 radiation-related toxicity, compared to GG or GA genotypes	21286719
rs861539	XRCC3		NSCLC	Resistance	rs861539 CC genotype was related to more resistant to platinum-based chemotherapy when compared patients with CT or TT genotypes (P = 0.009)	27248474, 23940523, 22374424, 22152690
			Ovarian cancer	Risk	rs861539 Thr/ Thr genotype associated with increased risk of death compare to Met/Met genotype	22938418
rs861539, rs1799794 and rs861530	XRCC3		Gastric cancer	Survival	rs861539, rs1799794 and rs861530 were associated with survival	21347786
XRCC3 -316A>G	XRCC3		Mesothelioma	Toxicity	XRCC3 -316A>G was found to associated with treatment-related toxicity.	22982660
rs1051685 and rs6941	XRCC5		Lung cancer	Toxicity	rs1051685 and rs6941 of XRCC5 were associated with hematologic toxicity	26358256
rs240966, rs456865, rs240969, rs3218573	REV1, REV3, and REV7:	Translesional Repair	NSCLC	Response, Toxicity	G/G genotype of rs240966 and rs456865 was associated with higher grade 3 or 4 gastrointestinal toxicity. rs240969 and rs3218573 were significantly associated with the treatment responses.	26278154
rs3792136	REV1		NSCLC	Risk	rs3792136 was associated with increased risk of mortality (P=0.030) .	26611653

rs3087403, rs462779	REV1		Osteosarcoma	EFS, OS	rs3087403 had significantly shorter EFS and OS (p = 0.004, p < 0.001 respectively). rs462779 was also significantly associated with shorter OS (p<0.001) and shorter EFS (p = 0.003) of cisplatin-based chemotherapy.	25748439
POLB P242R	POLB		Lung cancer	OS	POLB P242R was associated with OS (P = 0.018) in 456 lung cancer patients treated with platinum-based agents.	17855454
rs3213801	POLK		NSCLC	Survival	Patients with rs3213801 AA genotype had a shorter survival compared with AG or GG genotype (P= 0.010) .	26611653

1.3.4.3 Additional genetic variants identified by genome-wide analysis studies (GWAS)

Genome--wide gene expression or polymorphism analyses studies (GWAS) are approaches to rapidly scan the complete genome or transcriptome to identify highly significant and actionable genetic variations associated with a target disease or condition [507, 508].

GWAS studies that have so far identified gene expression patterns and SNPs with sensitivity to platinum drugs are listed in **Table 1.7** along with major details [509-513]. A GWAS study in 1,244 EOC patients treated with carboplatin and paclitaxel combination therapy identified two SNPs (rs7874043 and rs72700653) in TTC39B which were associated with PFS. On the other hand, genome-wide association scan in 334 NSCLC patient's cohort and 375 patients as validation cohort identified a SNP rs2838566 located at 21q22.3. Minor A allele was found to be significantly associated with increased risk of liver injury. Additionally, rs13014982 at 2q24.3 and rs9909179 at 17p12 were also found to be associated with myelosuppression in NSCLC patients [514, 515]. Additionally, another GWAS study explain SNP (rs11138019) association with the expression of gene ABCD2 along with carboplatin and cisplatin response [516]. Associations between NSCLC survival and SNPs were identified by GWAS study in Chinese populations. Three SNPs rs7629386 at 3p22.1, rs969088 at 5p14.1, and rs3850370 at 14q24.3 were associated with worse NSCLC survival while two SNPs rs41997 at 7q31.31 and rs12000445 at 9p21.3 were associated with better NSCLC survival. Furthermore, rs7629386 at 3p22.1 (CTNNB1) and rs3850370 at 14q24.3 (SNW1-ALKBH1-NRXN3) were associated with survival in Caucasian population [517]. Cell-based GWAS approach have been identified single nucleotide polymorphisms associated with platinum susceptibility which were then evaluated in head and neck cancer patients treated with platinum-based therapy. Two SNPs, rs6870861 and rs2551038 were associated significantly with overall response and expression of two important organic cation/anion transporters genes (SLC22A5 and SLCO4C1) which are involved in platinum uptake and clearance [518]. However, these LCLs are derived from

normal healthy individuals and therefore do not represent actual variations of cancer patients. Therefore, in vitro findings using these cell lines cannot be reliably validated using patient data ([518]. Similar approach also discovered SNP rs1878022 in the chemokine-like receptor 1 (CMKLR1) which was associated with poor OS in 327 advanced-stage NSCLC patients who received platinum-based chemotherapy[519]. GWAS study on SCLC patient cohort identified 3 loci, 20q13.2 (rs4809957G >A), 22q12.2 (rs36600C >T) and 5p15.33 (rs401681C >T) which were significantly associated with the survival time of SCLC patients. Hazard ratio (HR) for patients with the rs4809957 GA or AA genotype was 0.80 (P=0.0187) and 0.73 (P=0.0263) compared with the GG genotype and T allele for rs36600 or rs401681 was 0.78 (P=0.0199) and 1.29 (P=0.0047) respectively compared with the CC genotype [520]. Another study in SCLC identified association between rs1820453 T>G within the promoter region of YAP1 on chromosome 11q220 and rs716274 A>G in the region of downstream of DYNC2H1 (11q22.3) with small-cell lung cancer survival. Functional analysis showed that the rs1820453 T>G change creates a transcriptional factor binding site and downregulates YAP1 expression [521]. GWAS in LCLs for cisplatin cytotoxicity identified rs11169748 and rs2440915 SNPs in DAPK3 and METTL6 which were also associated with OS in lung cancer patients [522, 523]. GWAS approach on brain tumor had identified genetic variations in ACYP2 associated with cisplatin-related ototoxicity (rs1872328: $P = 3.9 \times 10^{-8}$; hazard ratio = 4.5) in brain tumors in children population [524].

Epstein-Barr Virus (EBV)-transformed human lymphoblastoid cell lines (LCLs) are used as model systems to identify germ-line genetic variations and gene expression associated with anticancer drug response. Using Epstein-Barr Virus (EBV)-transformed human lymphoblastoid cell lines (LCLs) as model system, SNPs in a number of genes have been identified as candidates for platinum-drug and paclitaxel response: The International HapMap Project is an organization that developed a panel of EBV-transformed LCLs (Hapmap panel)

from populations with African, Asian and European ancestry with a goal to create a publicly available catalog of common patterns of human genome variations including SNPs and gene expression [525]. Genome-wide association analysis with carboplatin and cisplatin cytotoxicity (IC_{50}) in HapMap LCLs with European (CEU), African (YRI) and Asian ancestries also been reported [526]. Association of SNPs and gene expression levels with carboplatin-induced cytotoxicity among 89 HapMap LCLs belonging to African ancestry (YRI) has been studied and results was validated by using 377 ovarian cancer patients receiving at carboplatin-based chemotherapy. SNP rs1649942 could be replicated in an independent LCL set and was also found significantly associated with decreased PFS in ovarian cancer patients [527]. Another Genome-wide meta-analysis of SNPs was associated with carboplatin- and cisplatin-induced cytotoxicity among 608 LCLs representing world population panels of diverse ancestry. Results showed a rs7572081 located in intronic region of the gene NBAS (neuroblastoma amplified sequence) and was most significantly associated with carboplatin cytotoxicity (P=5.1 × 10-7). Additionally, rs7210837 was significantly associated with cisplatin IC50 and rs244903 (located in the first exon of RARS) associated with carboplatin IC50. rs10138824 (located in an intron of MPP5-membrane protein, palmitoylated 5), rs8008724 (located in an intron of EIF2S1- eukaryotic translation initiation factor 2, subunit 1 alpha) and rs10431718 (located in an intron of FAM71D-family with sequence similarity 71, member D) associated with IC50 of both drugs and the expression of BCL2, GSTM1 (glutathione S-transferase mu 1) respectively. Furthermore, GSTM1, GSTT1, ERCC2 and ERCC6 were found associated with platinum drug response. [528].

Table 1.7 SNPs found significantly associated with Platinum drug chemotherapy outcome in GWAS studies

SNP ID (Gene symbol)	Cancer Type	Associated Platinum-drug Phenotype	PubMed ID (s)
rs7874043, rs72700653 (TTC39B)	Ovarian cancer	PFS	26840454
rs201023017 (SLC9A9), rs66696671 (TIAL1), rs10674174 (PCDH20), rs1525599 (LRP1B), rs2748151 (CDH4), rs12025262 (ZNF731P), rs150303591 (FRAS1)	Ovarian cancer LCLs	Associated with cytotoxicity in LCLs developed form EOC patients receiving carboplatin combination therapy	27047539
rs1649942	Ovarian cancer	PFS	21705454
rs2838566, rs13014982, rs9909179	NSCLC	rs2838566 A allele was significantly associated with increased risk of liver injury	26100964, 25823687
rs11138019 (ABCD2)		Gene expression of ABCD2; carboplatin and cisplatin response.	24739237
rs7629386 (CTNNB1), rs969088, rs3850370, rs41997, rs12000445	NSCLC	Worse NSCLC survival	22872573
rs6870861 (SLC22A5), rs2551038 (SLCO4C1)	Head and neck cancer	rs6870861 was associated significantly with overall response and expression of SLC22A5, which are involved in platinum uptake and clearance.	21497773
rs1878022 (CMKLR1)	NSCLC	Associated with poor OS in advanced-stage NSCLC patients who received platinum-based chemotherapy	21483023
rs4809957, rs36600, rs401681	SCLC	rs4809957 GA or (HR=0.80; P = 0.0187) and AA genotype (HR=0.73; P = 0.0263) compared with the GG genotype. Patients with rs36600 T allele (HR=0.78; P = 0.0199) compared with the CC genotype. Patients with rs401681 T allele of rs401681 (HR=1.29; P = 0.0047) compared to CC genotype.	25415319
rs1820453 (YAP1), rs716274 (DYNC2H1)	SCLC	rs1820453 T>G within the promoter region of YAP1 associated with small-cell lung cancer survival.	21118971
rs11169748 (DAPK3), rs2440915 (METTL6)	Lung cancer	Associated with OS in lung cancer patients	21775533
rs7572081 (NBAS), rs7210837, rs10138824 (MPP5), rs8008724 (EIF2S1), rs10431718 (FAM71D), rs244903 (RARS)	LCLs	Associated with carboplatin cytotoxicity in LCLs	21844884
rs1872328 (ACYP2)	Brain tumor	rs1872328 associated with cisplatin-related ototoxicity (P = 3.9 × 10(-8) in brain tumors in children population.	25665007

1.4 Paclitaxel chemotherapy

1.4.1 Introduction

Taxol is a microtubule binding, widely used chemotherapeutic agent [46]. It was first isolated from the bark of the yew tree (*Taxus brevifolia*) in 1967 by Monroe Wall and Mansukh Wani[529]. In 1979, Peter Schiff and Susan Horwitz discovered that paclitaxel stimulated microtubule polymerization [530]. Major members include paclitaxel and docetaxel. Basic structure of taxanes. **Figure 1.12** shows the basic structure of Taxanes along with differences in paclitaxel and docetaxel based on the moieties attached to the sites marked R1 and R2 in the basic structure [531]. paclitaxel (Empirical formula: $C_{47}H_{51}NO_{14}$; Molecular weight: 853.9 g/mol; Average mass: 853.9 Da; Trade names: Abraxane, Taxol, Onxol, Nov-Onxol) was approved for clinical use in 1995 and it is now widely used to treat various cancers including breast and ovarian cancer, non-small-cell lung cancer and Kaposi's sarcoma [532]. paclitaxel is a mitotic inhibitor that stabilizes microtubules. The stabilized microtubules interfere with the normal breakdown of microtubules during cell division leading to induction of apoptosis [532-538].

However, the response rate for paclitaxel based therapy is 65%, however tumor recurrence occurs in almost all patients approximately after 15 months [46]. Drug resistance is the principal cause for 90% of the deaths among EOC patients [539]. Furthermore, side effects such as severe hypersensitivity reactions (sHSR), hematological toxicity mainly severe neutropenia and peripheral neuropathy (PNP) are the major challenges for this drugs based chemotherapy [531, 540].

Lack of reliable biomarkers that would enable prediction of response or association with drug related toxicity is another challenge for developing paclitaxel individualized therapy. Identification of genes and polymorphisms associated with paclitaxel treatment outcome is the major goal for developing new therapeutic strategies with maximum drug efficacy along with decreasing drug related toxicity. Here we explore how differential expression and/or activity of genes are involved

in paclitaxel pathway and presence of SNPs can have an impact on treatment outcome and toxicity in cancer patients being treated with paclitaxel.

Understanding the pharmacogenomics of paclitaxel will provide an opportunity to identify likely responders along with poor responders, non-responders and patients with adverse effects which will help to provide better treatment.

1.4.2 Mechanism of action of paclitaxel

Paclitaxel is administered (175mg/m2) in combination with carboplatin (AUC 5–7.5) every 3 weeks for 6 cycles intravenously for EOC and NSCLC patients [43, 45, 58, 541-545]. Paclitaxel diffuses through the small openings on the microtubule surface or microtubule lattice fluctuations and binds to β-tubulin on the inside surface of the microtubule resulting in a conformational change in tubulin form (**Figure 1.13**) [546]. This alteration enhances microtubule assembly, inhibits depolymerisation and stability followed by mitotic arrest resulting from lack of microtubule bundling which eventually leads to apoptotic cell death (**Figure 1.13**) [532-538]. In addition, after entering the body, paclitaxel is metabolized by cytochrome enzymes CYP3A and CYP2C [547-550]. The major metabolites of paclitaxel are 6-α-hydroxypaclitaxel and p-3'-hydroxypaclitaxel which are formed by CYP2C8 and CYP3A4/5 metabolism, respectively (**Figure 1.12**) [551, 552]. Taxol metabolism was studied using membrane fractions from Hep G2 cells contain several human cytochrome P450 enzymes. Only P450 2C8 formed 6 alpha-hydroxytaxol and 3A3 and 3A4 was catalyzed Metabolite B formation. The correlation between hepatic 2C8 protein content and 6 alpha-hydroxytaxol formation was found to be high (r2 = 0.82) (Rahman et al. 1994). The role of individual modifications was then investigated by high-pressure liquid chromatography/mass spectrometry using human liver microsomes and recombinant P450 expressed in Ad293 cells [547-549, 549, 550]. Notably, these metabolites do not have appreciable antitumor activity [553].

Paclitaxel is excreted (70–80%) in bile as metabolites or as the parent drug by adenosine triphosphate- (ATP) binding cassette multidrug transporters including P-glycoprotein (P-gp or

ABCC1) and multidrug resistance protein 2 (MRP-2 or ABCC2). Renal clearance occurs only about 5–10% of drugs [547-550].

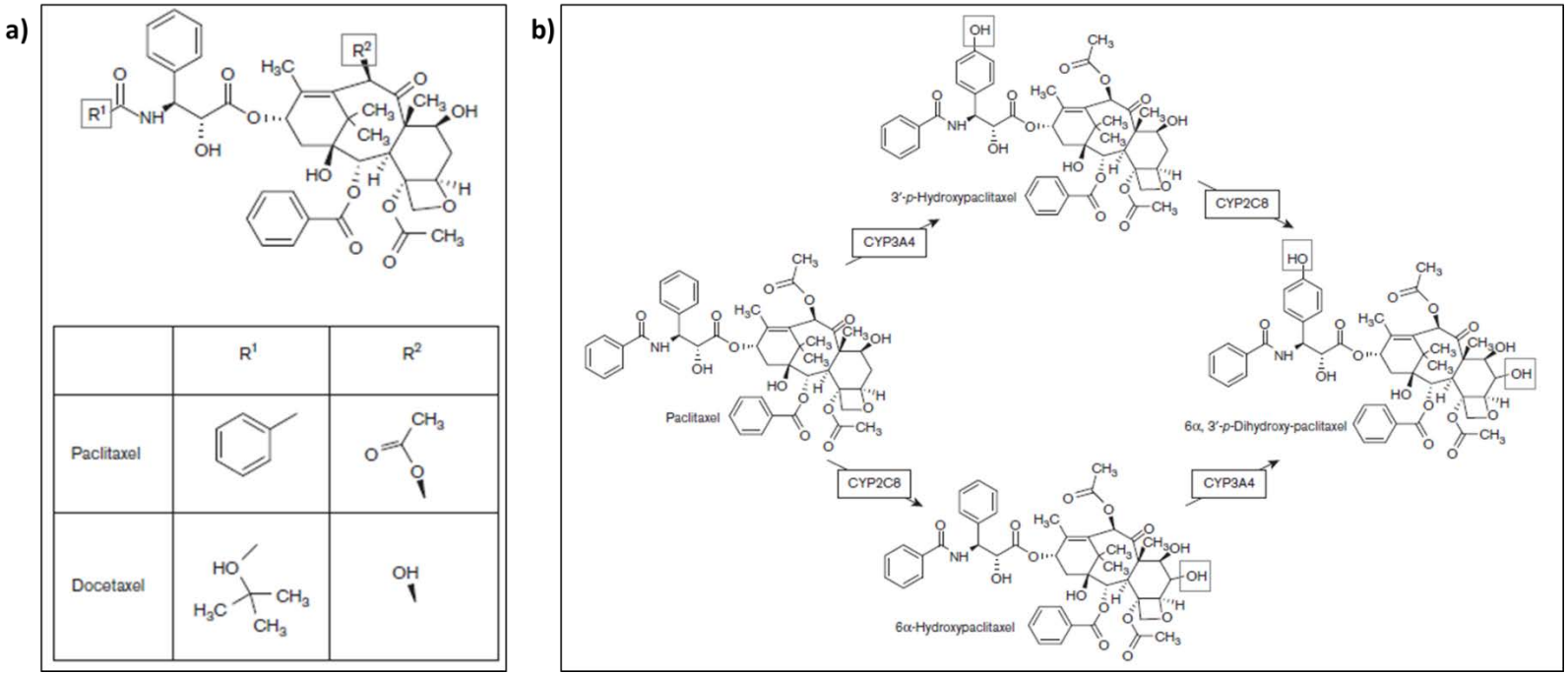


Figure 1.12 a) Basic structure of taxanes showing differences in paclitaxel and docetaxel (R^1 and R^2) [531] b) Major metabolites of paclitaxel with the enzymes involved [553].

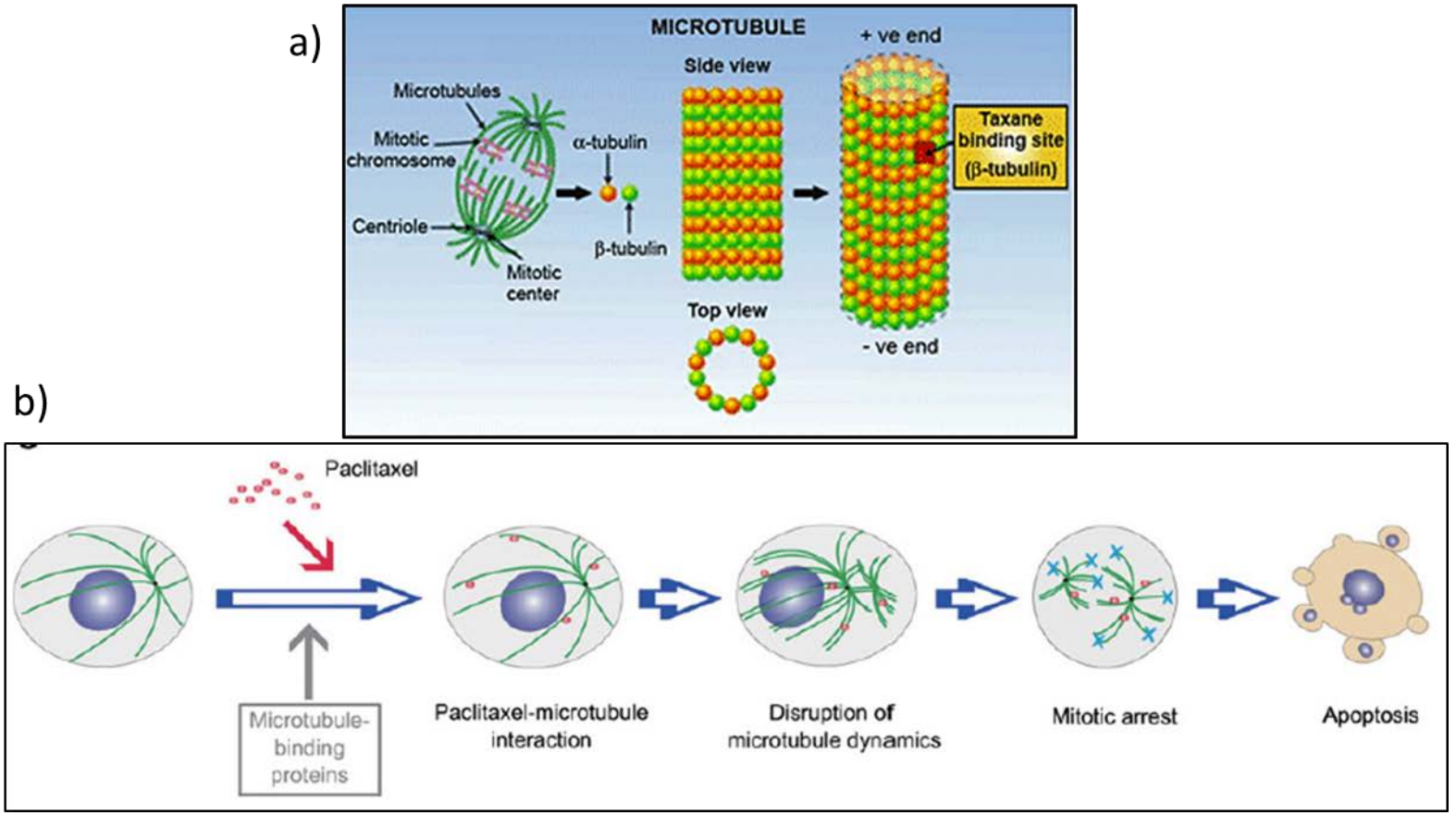


Figure 1.13 Mechanism of action of paclitaxel.

Figure shows a) binding to tubulin and b) overall schema of events leading up to cellular sensitivity to paclitaxel [532]

1.4.3 Metabolic (PK/PD) pathway of paclitaxel

Transporters are involved in the regulation of intracellular drug concentration for effective cytotoxic activity of paclitaxel [554]. paclitaxel is a substrate for several cell membrane transporting enzymes. paclitaxel enters into the cells through OATP1B3 (SLCO1B3) influx transporter. Polymorphism in this gene were found to be associated with paclitaxel pharmacokinetics [555, 556]. Additionally, OAT2 (SLC22A7) is highly expressed in human liver and was found to transport paclitaxel in vitro [557]. On the other hand, ATP-binding cassette (ABC) transporters, which include 48 proteins in humans, is involved in paclitaxel transport. Studies has been shown that inhibition of ATP-binding cassette sub-family B member 1 (ABCB1) or P-gp is associated with increased level of paclitaxel bioavailability [558-560]. Furthermore, it has been reported in several studies that ABCB1 was overexpressed in platinum resistant ovarian cancer cells, human lung carcinoma clonal cell line and also in other paclitaxel-resistant cell lines [539, 561]. Non-small cell lung carcinoma (NSCLC) patients receiving paclitaxel therapy with no or low expression of MDR-1/P-gp/ABCB1 showed better response compared to patients with increased MDR-1/P-gp/ABCB1 expression ($p<0.05$) [562]. In ovarian cancer, MDR-1/P-gp overexpression was also found to be significantly associated with poorer paclitaxel response and survival ($p=0.004$) [563, 564]. The multi-resistance-associated protein (MRP) family of transport proteins or ABCC (adenosine triphosphate-binding cassette C group) transporters is another class of drug efflux pumps which include ABCC1, ABCG2 and ABCC2 that are involved in paclitaxel transport as well as development of resistance against taxanes [168, 550, 565-569]. paclitaxel related neurotoxicity was associated with ABCB1, CYP2C8 and CYP3A5 polymorphisms [570-573].

CYP2C8 and CYP3A4 are key enzymes involved in the metabolism of paclitaxel. CYP2C8 metabolizes paclitaxel to the primary metabolite 6α-hydroxypaclitaxel whereas, two minor metabolites 3'-p-hydroxypaclitaxel and 6α, 3'-pdihydroxypaclitaxel are produced by CYP3A4. Extensive inter-individual variations in CYP genes influence paclitaxel treatment response [531, 574-

[576]. Furthermore, polymorphisms and gene expression changes in pregnane X receptor (PXR), a nuclear receptor involved in CYP enzymes induction and molecular regulation of CYP3A, may also contribute to drug resistance to chemotherapeutic agents in cancer patients [577].

Tubulin and its binding capacity with paclitaxel is a major player in paclitaxel drug resistance [578]. Altered expression levels of microtubule binding proteins (MBPs) are associated with paclitaxel drug response [579-582]. For example, microtubule-associated protein (MAP) 7 domain-containing protein 3 plays an integral role in breast cancer growth and metastasis [583-585]. Sub-categories MBPs include canonical MBPs, microtubule-destabilizing proteins, microtubule plus end-tracking proteins, and noncanonical MBPs. Canonical MBPs are structural MAPs and bind to the side of microtubules to stabilize it by promoting the polymerization and formation of microtubule bundles through MAP phosphorylation. MAP2 and tau are the member of this family [586]. MAP2 promotes paclitaxel sensitivity in breast cancer cell lines [587]. Knockdown of tau in breast cancer cells increases cancer cell sensitivity to paclitaxel [588]. Furthermore, studies reported that low concentration of tau is associated with increased paclitaxel binding to microtubules compare to the presence of higher levels of tau. So, low tau expression was found to be associated with the greater response to treatment whereas, high levels of tau was associated with residual tumor and resistance to treatment [589-592]. Additionally, overexpression of MAP4 increased microtubule polymerization and increased taxane binding with microtubule which resulted in increased sensitivity to the drugs [593, 594]. Microtubule-destabilizing proteins are important regulators of microtubule dynamic instability by restoring free α/β-tubulin heterodimers into a ternary complex, resulting in microtubule de-polymerization which eventually promotes the formation of spindle microtubules [595, 596]. Stathmin is the most important member of this family. Elevated expression of stathmin plays a role in cancer susceptibility to paclitaxel treatment [597, 598]. Overexpression of stathmin in breast cancer cells significantly decreases the binding of paclitaxel to microtubules and causes paclitaxel resistance, whereas, functional knockdown of stathmin using siRNA results in increased sensitivity

to paclitaxel [599-602, 602]. p53 regulates the expression of the MAP4 and statin [593, 594, 603-605]. Microtubule plus end-tracking proteins (+TIPs) accumulate at the plus ends of growing microtubules and modulate microtubule dynamics and functions. Members of the end-binding protein (EB) family are master regulators of the dynamic +TIPs network. EB1 regulates paclitaxel-induced mitotic arrest and apoptosis. Expression of EB1 is found to be associated with response to paclitaxel-based chemotherapy in breast cancer patients [606, 607]. CLIP-170 is another member of the +TIPs family that is found to be associated with breast cancer cell sensitivity to paclitaxel. Another member, Mitotic centromere-associated kinesin (MCAK), shows association with paclitaxel resistance through microtubule detachment from centrosomes [608, 609].

Non canonical MBPs are involved in the regulation of cell proliferation and survival and are also associated with paclitaxel sensitivity. Parkin is an important member of this sub-category. Higher expression of Parkin is associated with a better response to paclitaxel-containing chemotherapy in breast cancer [610]. Survivin, is another member that locates to the mitotic spindle during mitosis. Upregulation of survivin is associated with paclitaxel resistance in head and neck cancer breast cancer cells [611, 612]. Lower expression of Spindle genes MAD2 and BUBR1 in breast cancer cell lines shows resistance to paclitaxel [613].

Paclitaxel also has important effects on immune cells including macrophages, dendritic cells (DCs), natural killer (NK) cells, effector T-cells, regulatory T-cells (Tregs), and B-cells which eventually trigger antitumor immune response. paclitaxel stimulates macrophages, which cause cytotoxicity against tumor cells and start anti-tumor immune response [614]. In addition, paclitaxel also downregulates the expression of the anti-apoptotic molecule Bcl-2 in and upregulates expression of the pro-apoptotic member Bax, which leads cells towards apoptosis [615]. Furthermore, multiple translation factors along with gene-related oxidative stress (UGT1A6, MAOA, and CYBA), glycolysis (ADH1A, HK1, and ENO3) and glutathione metabolism are mainly responsible for paclitaxel resistance [616-618]. **Figure 1.14** is a modified version of PharmGKB pathway that

represents genes involved in metabolism and transport of paclitaxel and docetaxel, and the downstream effects of the drugs [619].

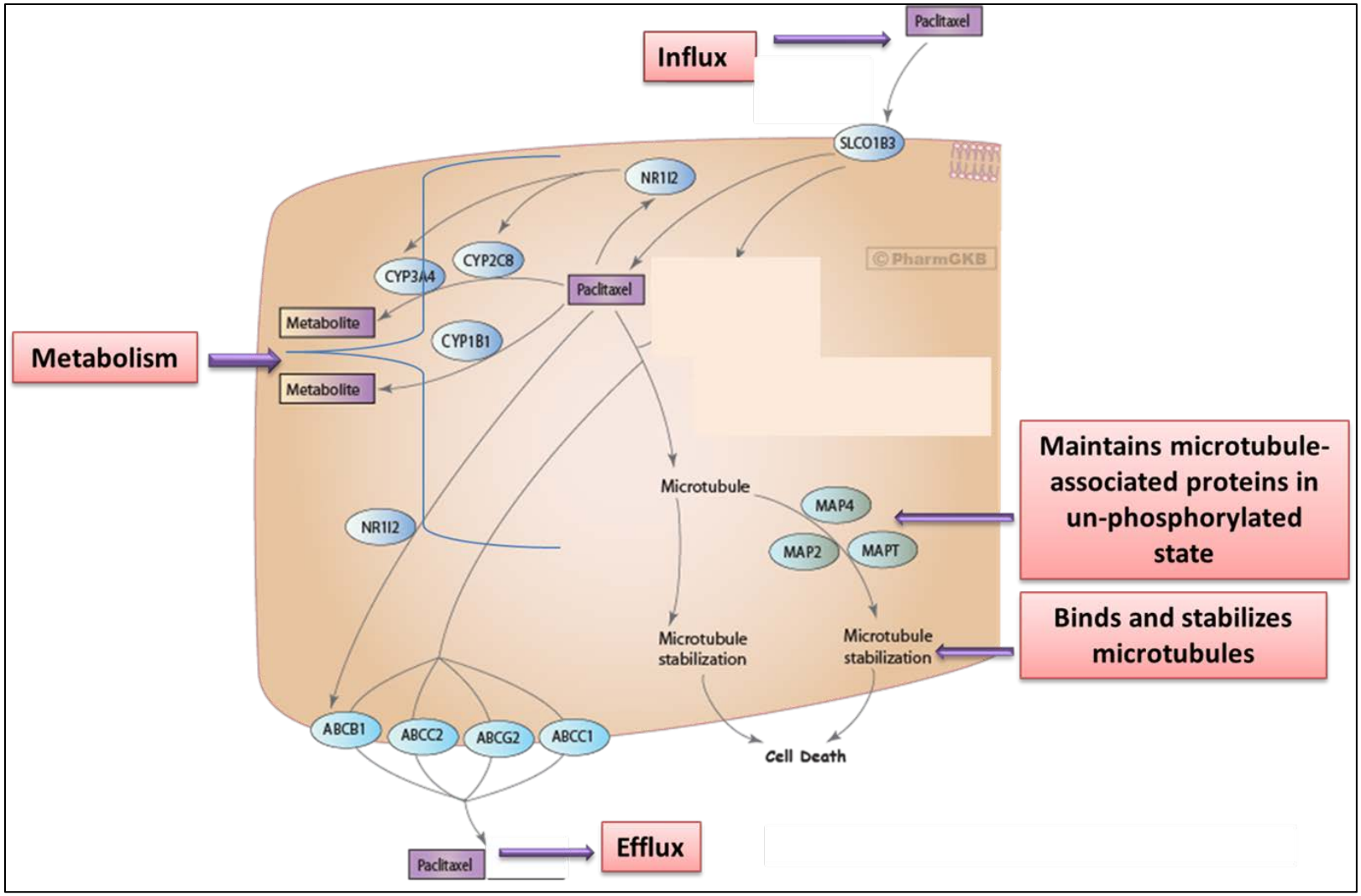


Figure 1.14 Metabolic pathway of taxanes.
The pathway was derived from the PharmGKB database [619]

1.4.4 Pharmacogenomics of paclitaxel (PK/PD) pathway genes

Differential gene expression and polymorphisms in genes involved in paclitaxel metabolism including influx and efflux transporters, pharmacodynamics and therapeutic targets may be associated to the inter-individual variability in response and toxicity. Here we report the relevant genetic variations in the paclitaxel-drug pathway and evaluate their association with chemotherapy. We performed a thorough and comprehensive study of the literature on the genes involved on taxane or paclitaxel-drug based chemotherapy. Our goal was to evaluate all the studies investigating association between gene expression changes and genetic polymorphisms in taxane or paclitaxel pathway genes and clinical response to paclitaxel therapy One of our criteria for identifying the genes to be selected for the study was based on the pathway information available from the resources providing information on key pathways, e.g. KEGG (Kyoto Encyclopedia of Genes and Genomes), GO (NCBI's Gene Ontology database), PharmGkb (The Pharmacogenomics Knowledgebase) and PGRN's (Pharmacogenomics Research Network) drug-specific pathway information.. Care was taken to ensure uniformity with respect to gene names and SNP IDs based on scientifically accepted notations and nomenclatures.

The following segment presents the salient genes and genetic variations that have been demonstrated to be associated with response to paclitaxel chemotherapy, as well as toxicity.

1.4.4.1 Pharmacogenomics of efflux-influx transporter and drug metabolizing (PK) genes

The organic anion transporting polypeptide (OATP)1B3 or SLCO1B3 is a key influx transporter for paclitaxel [556]. Polymorphisms within this gene have been reported with functional consequences [555]. Two polymorphisms in SLCO1B3, rs4149117 (c.334 T>G, Ser112Ala) and rs7311358 (c.699 G>A, Met233Ile) were associated with grade 3/4 anemia (P=0.002) in NSCLC patients. As shown in **Table 1.8**, rs7311358 (c.699 G>A) was also associated with overexpression of SLCO1B3 resulting significantly decreased uptake of paclitaxel compared with the wild-type [620].

The OS time and disease-free survival (DFS) were higher in patients with lower expression of ATP-binding cassette subfamily B member 1 (ABCB1) [621-623]. ABCB1 polymorphism rs2032582 (c.2677 T>G/A) was found to be associated with inferior PFS (hazard ratio=1.49, P=0.017) in NSCLC patients [620, 624]. Other studies also reported that ABCB1 polymorphisms rs1128503 (1236C>T), rs2032582 (2677G>T) and rs1045642 (3435C>T) have been found to be associated with paclitaxel response in various cancers including ovarian, lung prostate and breast cancer [247, 570, 622, 625-631, 631-634, 634, 635]. ABCB1 polymorphism rs2032582 (c.2677 T>G/A) was also found associated with grade 3 or 4 hematological and gastrointestinal toxicities in ovarian cancer patients [247]. Additionally, the 2677 GG genotype showed a significant association with paclitaxel resistance (P = 0.04) [636]. The ABCB1 polymorphism 3435C>T was associated with increasing risk to develop neuropathy (p=0.09) [571]. Another study in metastatic breast cancer patients treated with paclitaxel monotherapy showed that ABCB1 3435 C>T was associated with lower disease (p = 0.025) as well as shorter OS (P = 0.026). A study reported that the synonymous coding SNP rs1128503 (ABCB1; Gly412Gly; C1236T) was found associated with increased risk of anemia (p = 0.023) [249]. Another SNP p.Ser894Ala/Thr in ABCB1 was found associated with the risk of disease progression (p = 0,010) in ovarian cancer patients receiving paclitaxel/cisplatin chemotherapy [637]. The SNP rs2229109 (G1199T/A) in the ABCB1 gene was also shown to be associated with paclitaxel resistance in ovarian cancer patients treated with paclitaxel and carboplatin [638]. The ABCA1 SNP rs4149313 showed significant (p=0.03) association with thrombocytopenia [639].

Among SNPs in the ABCC2 gene, rs12762549 (ABCC2, C > G) was associated with decrease risk of anemia (p = 0.004) in ovarian cancer patients. rs2073337 (ABCC2, A > G) and rs1695 (ABCC1, A > G) was associated with colony stimulating factors (CSF) p = 0.039; p = 0.017, respectively), while rs2074087 (ABCC1, G > C) was associated with (p = 0.011) use of erythropoiesis stimulating agents (ESAs) [249]. rs2725264 in ABCG2 was found to be associated with OS in NSCLC receiving taxane-based chemotherapy (P=0.041)[238], while the non-synonymous variant in ABCG2,

rs2231142 (C421A encoding Q141K, Gln141Lys), was associated with longer median PFS (p=0.041) and a reduced risk of disease progression (p=0.022) in NSCLC patients [236].

CYP2C8 polymorphisms were shown associated with reduced metabolizing activity. Two Polymorphisms CYP2C8*3 (R139K; K399R) and CYP2C8*4 (I264M) are common polymorphisms in Caucasian population and associated with paclitaxel clinical response [640]. Patients with heterozygous for CYP2C8*3 showed lower clearance of paclitaxel [570, 641, 642]. Another study reported that two polymorphisms CYP2C8*2 and CYP2C8*3 associated with two-fold higher Km due to lower activity and lower intrinsic clearance of paclitaxel [574]. One study found the genetic variants CYP2C8*3, and CYP3A5*3 to be associated with paclitaxel-induced toxicity. CYP2C8-HapC showed association with lower level values of both leukocytes and neutrophils ($p < 0.05$) than patients with the wild-type genotype. Combining the two genotypes CYP3A5*3/*1 and CYP2C8-HapC (rs1113129 G/C) showed significant correlation with both leukopenia and neutropenia ($p = 0.01$) [643]. Furthermore, in a study in Chinese epithelial ovarian cancer patients treated with paclitaxel/carboplatin therapy, CYP3A5*3 (rs776746 G/C) was found to be associated with paclitaxel toxicities such as myelo-suppression where CYP3A5*3/*1 genotype showed higher risk of developing leukopenia ($p < .001$) and neutropenia ($p < .001$) [644]. One study reported that CYP3A4*1B was found to be associated with paclitaxel pharmacokinetics in ovarian cancer patients [570]. Another CYP3A4 polymorphism rs4986910 (CYP3A4, T > C) was found to be associated with increased risk of thrombocytopenia ($p = 0.025$) [249]. Two other CYP3A4 variants, rs2740574 (CYP3A4*1B) and rs35599367 (CYP3A4*22), were found associated with a higher CYP3A4 activity and clinical response of ovarian cancer patients treated with first-line paclitaxel and cisplatin or carboplatin chemotherapy. Patients carrying the CYP3A4*1BG allele showed decreased mean survival rate and lower OS when compared to the AA genotype patients ($p = 0.010$) [645]. Another study reported that polymorphism CYP3A4*16B was associated with both reduced 3'-p-hydroxylation of paclitaxel and increased levels of 6alpha-hydroxypaclitaxel. CYP3A4*16B

showed lower 3'-p-hydroxypaclitaxel metabolites production (P = .04) and a 2.4-fold higher production of 6alpha-hydroxypaclitaxel to paclitaxel (P < .001) compaed with wild-type (1/1) patients [646].

Table 1.8 Summary of SNPs in efflux-influx transporter and drug metabolizing (PK) genes associated with Paclitaxel chemotherapy.

SNP ID	Gene symbol	Gene function	Cancer Type	Associated Phenotype	Associated Genotype	PubMed ID (s)
rs2032582	ABCB1	Drug transporter	Ovarian cancer	Risk	p.Ser894Ala/Thr associated with on the risk of disease progression (p = 0.010).	25591549
rs4149313	ABCA1	Drug transporter	Lung cancer, ovarian cancer	Toxicity	rs4149313 was significantly (p=0.03) association with thrombocytopenia.	22759513
rs1045642	ABCB1	Drug transporter	Advanced solid tumours	Toxicity	rs1045642 was ssociated with increasing risk to develop neuropathy (P=0.09).	16950614
	ABCB1	Drug transporter	Breast cancer	OS	rs1045642 C>T was associated with lower disease (p = 0.025) as well as shorter OS (P = 0.026).	18836089
rs1128503	ABCB1	Drug transporter	Ovarian cancer	Toxicity	rs1128503 was associated with increased risk of anemia (p = 0.023).	25881102
rs2032582	ABCB1	Drug transporter	Ovarian cancer	Toxicity	rs2032582 was associated with grade 3 or 4 hematological and gastrointestinal toxicities.	19203783
rs2032582, rs1128503 and rs1045642	ABCB1	Drug transporter	Ovarian, lung prostate and breast cancer	Response	rs2032582 was found associated with inferior PFS (P=0.017) and GG genotype associated with paclitaxel resistance (P = 0.04) in NSCLC patients. Also associated with paclitaxel response in various cancers including ovarian, lung prostate and breast cancer.	23917080, 19203783, 18765553, 24810093, 21955855, 21883677, 21687948, 20944127, 19401306, 16803472, 16467099, 15901749 and 17062699

rs2229109	ABCB1	Drug transporter	Ovarian cancer	Rsponse	rs2229109 associated with paclitaxel resistance.	17828752
rs12762549	ABCC2	Drug transporter	Ovarian cancer	Toxicity	rs2725264 was associated with decrease risk of anemia (p = 0.004).	25881102
rs2231142	ABCG2	Drug transporter	NSCLC	PSF,Risk	Associated with longer median PFS (p=0.041) and a reduced risk of disease progression (p=0.022).	22112610
rs2725264	ABCG2	Drug transporter	NSCLC	OS	Associated with OS (P=0.041).	23689644
rs4149117, rs7311358	SLCO1B3	Drug transporter	NSCLC	Toxicity	c.334 T>G (Ser112Ala) was associated decreased uptake of paclitaxel and grade 3/4 anemia (P=0.002). c.699 G>A (Met233Ile) was associated with grade 3/4 anemia (P=0.002).	26641474
CYP3A4*16B	CYP3A4	Drug metabolizing enzyme	NSCLC, SCLC, breast cancer, other cancers	PK	Associated with both reduced 3'-p-hydroxylation of paclitaxel and increased levels of 6-alpha-hydroxypaclitaxel	16890579
rs2740574 (CYP3A4*1B)	CYP3A4	Drug metabolizing enzyme	Ovarian cancer	PK, Response	Associated with paclitaxel pharmacokinetics and clinical response of ovarian cancer patients. CYP3A4*1BG allele showed decreased mean survival rate and lower OS when compared to the AA genotype patients (p = 0.010)	23936594, 19143748
rs35599367 (CYP3A4*22)	CYP3A4	Drug metabolizing enzyme	Ovarian cancer	Activity, Response	Associated with a higher CYP3A4 activity and clinical response of ovarian cancer patients	23936594
rs4986910	CYP3A4	Drug metabolizing enzyme	Ovarian cancer	Toxicity	Associated with increased risk of thrombocytopenia (p = 0.025)	25881102

CYP3A5*3	CYP3A5	Drug metabolizing enzyme	Epithelial ovarian cancer	Toxicity	CYP3A5 *3/*1 genotype showed higher risk of developing leukopenia ($p < .001$) and neutropenia ($p < .001$). Associated with paclitaxel-induced toxicity such as myelo-suppression.	21702053, 26179145
CYP2C8*3	CYP2C8	Drug metabolizing enzyme	Ovarian cancer	Clearance	Associated with lower clearance of paclitaxel	20368717, 19143748, 17923851, 11668219
rs1113129	CYP2C8	Drug metabolizing enzyme	Ovarian cancer	Toxicity	rs1113129 G/C association with lower level values of both leukocytes and neutrophils ($p < 0.05$).	21702053

1.4.4.2 Pharmacogenomics of Microtubule binding protein, apoptosis and immune response (PD) genes

Among the genes involved in paclitaxel pharmacodynamics, low/negative class III β-tubulin expression was associated with significantly higher response rate for paclitaxel based chemotherapy in NSCLC patients (P<0.00001). Median survival time was longer for patients with low/negative expression of class III β-tubulin compared with patients with high/positive expression (P<0.00001) [647]. In addition, significantly increased MAP2 Expression in breast cancer patients showed complete response to neoadjuvant paclitaxel [587]. TUBB is one of the primary genes involved in the encoding of tubulin β subunits. Polymorphism of TUBB gene was found to be associated with both poor response and shortened OS to paclitaxel-containing chemotherapy in NSCLC patients [648]. Promoter polymorphisms -101, -112, and -157 in TUBB2A associated with 63-fold variation in b-tubulin IIa gene (TUBB2A) mRNA expression. These polymorphisms were beneficial, and protected patients from paclitaxel-induced peripheral neuropathy (HR, 0.62; 95% confidence interval (CI), 0.42–0.93; P = 0.021). Additionally, these polymorphisms were inversely correlated with paclitaxel-induced apoptosis level (P = 0.001) in lymphoblastoid cell lines confirming that higher TUBB2A gene expression conferred lower paclitaxel sensitivity [649].

Most important among the microtubule-destabilizing enzymes, high stathmin (STMN1) expression have been shown to be associated with poor prognosis in ovarian cancer patients treated with paclitaxel and platinum [602]. The promoter polymorphism rs182455 in STMN1 gene is associated with taxane outcome (**Table 1.9**). Patients with TT genotype showed longer PFS and the lower risk of early disease progression (p = 0.0154)[650]. Mitotic arrest deficient 1 (MAD1) is a mitotic spindle assembly checkpoint (SAC) protein plays an important role in metaphase arrest. The polymorphism rs1801368 (MAD1 1673 G>A) was associated with the therapeutic response of patients with ovarian cancer with paclitaxel based therapy. The GG patients showed a higher rate of response and cells showed higher percentage of mitotic arrest (p<0.05) [651].

Studies concerning TP53 gene reported that the Pro/Pro genotype of rs1042522 (p.Arg72Pro TP53) in p53 was associated with better progression free survival (PFS), higher response rate, and OS ($p = 0{,}008$) among ovarian cancer patients receiving paclitaxel/cisplatin chemotherapy [637, 652-656]. Polymorphisms in apoptotic genes were also found to be associated with paclitaxel response. These include as rs1061624 (TNFRSF1B), rs2279115 (BCL2), rs9904341 (BIRC5), and rs3769818 (CASP8) were significantly associated with OS in NSCLC patients receiving paclitaxel-cisplatin chemotherapy [657].

A study investigating the role of immune checkpoints genes polymorphisms with paclitaxel-cisplatin chemotherapy in NSCLC patients identified rs2297136T > C and rs4143815C > G in PD-L1 was significantly associated with better chemotherapy response, whereas rs2297136T > C was also associated with better OS [658, 659].

Table 1.9 Summary of SNPs in Microtubule binding, apoptosis and immune response (PD) genes associated with Paclitaxel chemotherapy.

SNP ID	Gene symbol	Gene function	Cancer Type	Associated Phenotype	Associated Genotype	PubMed ID (s)
rs1801368	MAD1	Microtubule assembly	Ovarian cancer	Response	rs1801368 was associated with therapeutic response of paclitaxel based therapy. The GG patients showed a higher rate of response and cells showed higher percentage of mitotic arrest (P<0.05).	23407047
rs182455	STMN1	Microtubule assembly	NSCLC	PFS, Risk	Patients with rs182455 TT genotype showed longer PFS and the lower risk of early disease progression (p = 0.0154)	26148901
rs2279115 (BCL2), rs9904341 (BIRC5), rs3769818 (CASP8), rs1061624 (TNFRSF1B)		Apoptosis	NSCLC	OS	Significantly associated with OS.	23973201
rs1042522	TP53	Apoptosis	Ovarian cancer	PFS, OS	rs1042522 was associated with better PFS, higher response rate, and OS (p = 0.008).	25591549, 23574945, 22331725, 16739339, 16364249, 15958617
rs2297136, rs4143815	PD-L1	Immune checkpoint genes	NSCLC	OS, Response	rs2297136T > C was associated with better OS; rs4143815C > G in PD-L1 was significantly associated with better chemotherapy response.	27181838, 27198292

1.4.4.3 Genome-wide association study (GWAS) genes

Genome-wide association study (GWAS) in 1,244 EOC patients treated with the carboplatin and paclitaxel showed two SNPs (rs7874043 and rs72700653) in TTC39B associated with PFS by interacting with the promoters of PSIP1, CCDC171 and an alternative promoter of TTC39B [523]. Another GWAS study in EOC patients treated with paclitaxel/carboplatin standard doses identified a number of SNPs rs4910232 (11p15.3), rs2549714 (16q23), and rs6674079 (1q22) and rs6674079 were significantly associated ($P \leq 1.0 \times 10(-5)$) with poorer outcomes [632]. Furthermore, a GWAS study on advanced NSCLC patients with squamous cell histology showed that tag SNPs rs4151510, rs4151465, rs9568036 in RB1 gene was associated with clinical response of first-line platinum-taxane. The patients with G/G genotype of rs4151510 had longer OS (p=0.018) [660]. An extensive GWAS study performed in 276 population panel LCLs and validated in 76 small and 798 non-small cell lung cancer (SCLC, NSCLC) patients treated with paclitaxel found a SNP rs1106697 on chromosome 7 to be associated with paclitaxel response in both LCLs and lung cancer patients (NSCLC and SCLC) ($p = 0.016$ and $p = 0.007$ respectively). The three SNPs rs1778335, rs2662411 and rs7519667 were associated with SCLC OS ($p = < 0.05$) [661]. Among other GWAS studies, one analysis showed association of SNPs in AIPL1 and BCR with sensory neuropathy in cancer patients [662]; rs1656402 in the EIF4E2 gene, rs1209950 in the ETS2 gene and rs9981861 in the DSCAM gene was found associated with poor OS in NSCLC patients [663]. A study performing analysis of single nucleotide polymorphisms (SNPs) located in the miRNA target sites and their role in paclitaxel-cisplatin chemotherapy in advanced NSCLC showed ETS2 rs461155A>G was significantly associated with decreased ETS2 mRNA expression[664]. **Table 1.10** summarizes the SNPs that were found significantly associated with paclitaxel chemotherapy in GWAS studies conducted so far.

Table 1.10 SNPs found significantly associated with paclitaxel drug chemotherapy outcome in GWAS studies.

SNP ID (Gene symbol)	Cancer/Cell Type	Associated Phenotype	Associated Genotype	PubMed ID (s)
rs7874043, rs72700653 (TTC39B)	EOC	PFS	rs7874043 and rs72700653 were associated with PFS	26840454
rs4910232, rs2549714, rs6674079, rs6674079	EOC	Response	significantly associated ($P \leq 1.0 \times 10(-5)$) with poorer outcomes	26152742
rs4151510, rs4151465, rs9568036 (RB1)	NSCLC	Response	associated with clinical response	25684524
rs1106697, rs1778335, rs2662411 and rs7519667	NSCLC, SCLC	Response, OS	rs1106697 was associated with paclitaxel response in both LCLs and lung cancer patients (NSCLC and SCLC). rs1778335, rs2662411 and rs7519667 were associated with SCLC OS ($p = < 0.05$)	23006423
rs1656402 (EIF4E2), rs1209950 (ETS2), rs9981861 (DSCAM)	NSCLC	OS	Associated with poor OS	21079520
rs461155 (ETS2)	LCLs	mRNA expression	rs461155A>G was significantly associated with decreased ETS2 mRNA expression	26893365

1.5 Research Study Objectives

1.5.1 Gaps in literature

Understanding the pharmacogenomics of response to carboplatin and paclitaxel chemotherapy is a pre-requisite to identify reliable genetic signatures of drug response and toxicity. Several studies, including GWAS, have investigated the association of differential expression and/or activity of genes involved in the PK/PD pathway due to SNPs with platinum drug-based treatment outcome and/or toxicity in various cancers and have identified several key pathway genes polymorphisms [227, 229, 665, 666]. However, a comprehensive pharmacogenomics-based evaluation of these genes to identify genomic markers predictive of efficacy/sensitivity is still lacking. However, most SNPs identified in such studies have not been validated using independent investigation.

One of the strategies to identify biomarkers associated with cellular sensitivity to carboplatin and/or paclitaxel is use of Epstein-Barr Virus (EBV)-transformed human lymphoblastoid cell lines (LCLs) as model systems. SNPs associated with sensitivity have been identified by genome wide analysis using LCLs that are part of International HapMap project [189, 191, 192, 667]. However, one of the biggest disadvantages of using these LCLs is that they were derived from normal healthy individuals and therefore do not represent variations in cancer patients. Secondly, these cell lines might not reflect gene expression profiles of the target patient subject. Furthermore, large-scale association studies conducted so far have not provided reliable and clinically applicable biomarkers of carboplatin/paclitaxel response in ovarian/lung cancer due to low sample sizes or lack of reproducibility [138, 186, 288, 668, 669]. Notably, no study so far has focused on the assessment of carboplatin + paclitaxel combination treatment response using *in vitro* model systems. Therefore, a systematic and comprehensive genotype-phenotype association study is required to understand the association between genetic variants and carboplatin + paclitaxel drug response in cancer treatment.

1.5.2 Objectives and Specific Aims

We hypothesize that we will identify a robust set of genetic variants and gene expression signatures significantly associated with drug response and treatment outcomes within *in vitro* cancer cell lines model systems as well as cancer patients undergoing carboplatin and paclitaxel-based chemotherapy. Thus, the objectives of this study were to: 1) Use LCLs derived from ovarian cancer patients to identify predictive markers of chemo-sensitivity; 2) Comprehensively and systematically evaluate association of gene expression and SNPs with treatment outcome in *in vitro* cell line-based models and clinical trials of ovarian and lung cancer patients.

Our objectives were achieved through the following specific aims:

Specific Aim 1. To create an *in vitro* cytotoxicity profile of EBV-transformed LCLs from epithelial ovarian cancer patients in response to carboplatin and paclitaxel as single agents and in combination.

> **Hypothesis:** LCLs from ovarian cancer patients demonstrate inter-patient variation in *in vitro* cellular sensitivity to carboplatin and paclitaxel.

Specific Aim 2. To evaluate gene expression levels and SNPs in key PK and PD pathway genes in epithelial ovarian cancer patients and correlate these with cytotoxicity data obtained from Aim1.

> **Hypothesis:** Variation in gene expression levels and the presence of SNPs in PK/PD pathway genes influence treatment outcomes in ovarian cancer patients.

Specific Aim 3. To determine correlation of cytotoxicity, gene expression and SNPs identified using Genome-wide association study with clinical response (progression free and OS) and toxicity (myelosuppression, gastrointestinal toxicity) in the ovarian cancer patients from which the LCLs are derived.

Hypothesis: *In vitro* cytotoxicity profiles, gene expression and SNPs are correlated with clinical outcome and toxicity in cancer patients treated with carboplatin/paclitaxel-containing chemotherapy.

Specific Aim 4: To identify genetic variations in platinum drugs and taxane pathway genes as predictors of outcome and toxicity in advanced non-small-cell lung cancer (NSCLC)

Hypothesis: SNPs within the genes involved in the metabolic pathways of taxanes and platinating agents are associated with clinical outcome and toxicity in NSCLC patients treated with carboplatin/paclitaxel-containing chemotherapy.

Long term goal: Successful completion of the project will generate a robust panel of genetic biomarkers of carboplatin and paclitaxel single-agent and combination treatment response that can serve as a first step towards the development of a precision medicine approach to develop pharmacogenomics-guided chemotherapy for maximum efficacy and minimum toxicity.

CHAPTER 2 PHARMACOGENOMIC MARKERS INFLUENCE THE VARIATION OF IN VITRO CHEMOSENSITIVITY TO CARBOPLATIN AND PACLITAXEL AS SINGLE AGENTS AND AS COMBINATION: A PATHWAY BASED APPROACH

2.1 Introduction

Ovarian cancer is the most lethal gynecologic malignancy in the United States (NCI SEER cancer statistics) [1]. An estimated 220,000 new ovarian cancer (OC) cases are diagnosed every year worldwide while around 140,000 women die from this disease [2]. Epithelial ovarian cancer (EOC) is the one of the most common gynecological malignancies and the fifth leading cause of cancer death among women in the United States [3]. EOC occurs primarily in middle aged or older women, rarely before puberty [4].

The standard treatment for 75% of EOC patients with advanced disease is initial debulking surgery followed by carboplatin-paclitaxel combination chemotherapy[5]. carboplatin [cis-diammine (1,1-cyclobutanedicarboxylato) platinum(II)] or Paraplatin was approved by FDA in 1989 for the treatment of advanced ovarian cancer [6]. carboplatin forms DNA inter-strand, intra-strand, and DNA-protein crosslinks which is attributed to the formation of DNA helix-distorting adducts that result in strand breakage followed by activation of DNA repair mechanisms that interfere with cell division, which ultimately results in apoptosis [7]. Some of the major genes involved in platinum pathway are influx and efflux transporters *SLC31A1, ABCC2, ATP7*; DNA repair genes *MSH6, MLH1, XRCC1, ERCC1, ERCC2, XPA*; and xenobiotic metabolism genes *MPO, SOD1, GSTM1, NQO1, GSTP1* [8-10]. paclitaxel is another chemotherapeutic agent, used generally in combination with platinum drugs for various cancers including lung and ovarian cancers [11]. Taxanes like paclitaxel block cell division by binding to α-tubulin that stabilizes the microtubules resulting in cell death [11, 12], a process regulated by microtubule- associated proteins *(MAPs) MAPT, MAP2 and MAP4* [13]. paclitaxel is transported into the cell through influx transporter *OATP1B3 (SLCO1B3)* and metabolized by *CYP3A4* and *CYP2C8* [14, 15] while the drug is effluxed out of the cell by ATP binding cassette *(ABC)* multidrug transporters *ABCB1, ABCG2, ABCC1* and *ABCC2.*

However, despite currently available therapeutic strategies, 5-year overall survival (OS) in EOC remains around 44.2% along with wide inter-patient variation in response [16]. Response rate is 10–

15% and overall survival is ~12 months in resistant patients [17]. 90% of the patients relapse after 18 months of treatment [18].

Though patients commonly develop resistance to these chemotherapeutic agents, the underlying mechanisms still remain unclear. Alterations in expression of key candidate genes involved in drug metabolism pathways and/or the presence of candidate SNPs may have a profound impact on treatment outcome/resistance and toxicity in ovarian cancer patients. However, there are not many reliable prognostic biomarkers for either carboplatin or platinum to help improve clinical response.

Epstein-Barr virus (EBV)-transformed Lymphoblastoid cell lines (LCLs) are immortalized cell lines generated from human normal lymphocytes through the infection of Epstein-Barr virus (EBV) [19]. EBV-transformed LCLs comprising of multi-ethnic panels representing multiple world subpopulations, including the International HapMap project [20-22] and 1000 genomes project [23], have been extensively used to identify genetic biomarkers for various chemotherapeutic agents using both Genome-wide association and candidate gene approaches [20, 24-26]. However, since these LCLs are derived from 'normal' healthy individuals, they do not truly represent the genetic architecture of EOC patients.

In the current study, we created EBV-transformed LCLs from cells derived from EOC patients, performed *in vitro* chemo-sensitivity assays to derive cytotoxicity profiles for carboplatin, paclitaxel and combination treatments, and conducted genetic association studies between *in vitro* drug response phenotypes, gene expression levels and genetic variations of drug pharmacokinetic (PK) and pharmacodynamics (PD) pathway genes to identify reliable genomic signatures of treatment outcomes.

2.2 Materials and Methods

2.2.1 Creation of EBV-transformed Ovarian cancer LCLs

Prior to initiation of chemotherapy, blood was collected following informed consent from ovarian cancer patients, diagnosed at the Mayo Clinic between 2000 and 2003. Epstein Barr Virus (EBV)-transformed LCLs were generated from patients and subjected to *in vitro* drug testing. All patients provided informed consent, including for passive and active follow-up, using protocols approved by the appropriate Institutional Review Board at the Mayo Clinic in Rochester, MN.

2.2.2 Cell Culture

All the cell lines were cultured in RPMI1640 media (Gibco) supplemented with 2 mM L-glutamine (Gibco) and 15% fetal bovine serum (HyClone) and were maintained in a humidified incubator at 37°C with 5% CO2. The cells were passaged every 2 to 3 days in order to maintain them in logarithmic growth phase. To confirm healthy growth, cell viability was measured using Countess Automated Cell Counter (Life Technologies) and cell line with >80% viability was used for further experiments.

2.2.3 Drugs

Carboplatin and paclitaxel were procured from Sigma Aldrich (St. Louis, MO). carboplatin was freshly prepared in PBS while paclitaxel was prepared in DMSO (Dimethyl sulfoxide) and stored in -20°C in aliquots for 8 weeks.

2.2.4 In vitro cytotoxicity assays

MTT (5-diphenyltetrazolium bromide) reagent (Sigma Aldrich, St. Louis, MO) was used to perform *in vitro* cytotoxicity assays on the cell lines treated with single-agent or combination regimens. Briefly, LCLs were plated in 96-well plate at seeding density of 2.5×10^5 cells/ml and incubated at 37°C overnight. Following 24 hour incubation, LCLs were treated with increasing concentrations of carboplatin and/or paclitaxel (in duplicate). Concentrations were 0, 5, 10, 20, 40, 80 and 128 uM for carboplatin and 0, 4.5, 7.5, 10, 20, 40 and 80 nM for paclitaxel treated as single agents. In combination treatment, individual drug doses were half (0.5) of the doses for each drug

used as single agent in increasing concentrations. Cells were incubated at 37C for 48hrs following treatment. Cell viability was determined 48hrs post-treatment by incubating the cells with MTT reagent followed by measurement of absorbance at 570 nm the using Synergy plate reader (BioTek, USA). The percent cell survival at each concentration and IC_{50} values and area under survival curve (AUC) were calculated as described in the statistical analysis sub-section.

2.2.5 Caspase-Glo 3/7 activity Assay

Apoptotic activity within the LCLs was determined 48 hours following treatment with carboplatin, paclitaxel, or combination, the using Caspase-Glo® 3/7 Assay (Promega, USA). Luminescence Intensity were determined as measures of caspase activity using Synergy plate reader (BioTek, USA). The caspase activity at each concentration was normalized to control.

2.2.6 Cell cycle analysis

Prior to plating for cytotoxicity and apoptosis experiments, an aliquot from each fresh cell line was collected to measure %S phase. Briefly, cells were stained with Propidium Iodide (PI) followed by assessment of cell cycle phases using BD FACSCalibur Flow Cytometer (BD Biosciences). FlowJo software was used to analyze the % of cells in different cell cycle phases (synthesis phase, growth phases).

2.2.7 SNP Genotyping

Patient germline DNA was isolated and genotyped on the Illumina Infinium 610K array, as previously described [27]. Genotypes were predicted by STRUCTURE [28] analysis to have greater than 80% European ancestry. The 1000 Genomes Project [23] was used as reference, imputation was completed with *mach* and *minimac* in a two-step process [29]. SNPs within carboplatin and paclitaxel drug pathway genes were extracted by using the web-based SNPNexus software.

2.2.8 RNA Isolation and Gene expression assays

Total RNA was isolated from LCLs before drug treatment using RNeasy Plus Mini Kit (QIAGEN, USA) according to manufacturer's protocol. RNA quantity and concentration were measured using

NanoDrop 2000 UV-Vis spectrophotometer (ThermoScientific, USA). The absorbance ratio between absorbances at 260 nm and 280 nm (A260/A280) was computed for assessing RNA sample purity. A260/A280 ratio between 1.8-2.1 was considered as highly pure RNA. Further, RNA Quality check was performed using Agilent Bioanalyzer (Agilent RNA 6000 Pico kit) and estimated by calculating RNA Integrity Number (RIN) with 2100 expert software version B.02.02. RIN greater than 7 was considered indicating good quality RNA. The total RNA was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to manufacturer's protocol.

Expression of key pharmacokinetic and pharmacodynamics pathway genes of carboplatin and paclitaxel was determined using the TaqMan® Low Density Array (TLDA) cards (Applied Biosystems, USA). Briefly, each TLDA card was custom designed with pre-loaded assays for measuring the mRNA expression of selected genes that includes influx and efflux transporters, DNA repair gene, detoxifying gene for carboplatin and genes for microtubule associated protein, transporters for paclitaxel, along with the housekeeping genes GAPDH and Actin β **(Table 2.1)**. Each TLDA card consists of eight separate loading ports which can fill into 48 separate wells for each sample. 24 different genes can be assayed in duplicates in a total of 384 wells per card. Therefore, each card could analyze the expression of 8 different samples. High-quality cDNA and equal volume of 2X TaqMan Universal PCR Master Mix was loaded in to the TLDA card along with 100 ul of the sample-specific PCR mix. To distribute reaction mix to the reaction wells properly, the TLDA cards were then centrifuged twice for 1 minute at 1200 rpm using a Sorvall Legend T Centrifuge that has Sorvall Custom Buckets with TLDA card holder. The cards were then sealed by the TaqMan Array Micro Fluidic Card Sealer and real-time quantitative polymerase chain reaction (qRT-PCR) was performed on microfluidic card thermal cycling block of Applied Biosystems 7900HT Fast Real-time PCR System (Applied Biosystems). Thermal cycling conditions were: initial denaturation of 10 minutes at 94.5°C followed by 35 cycles of: annealing

and extension of 2 minutes at 50°C, and denaturation at 30 seconds at 97°C and finally 1 minute at 59.7°C for 40 cycles. For every run, Ct (cycle threshold) values or the cycle number required for the fluorescent signal to cross the background level were determined for each gene and used for further analysis. Average Ct values representing the mRNA expression levels were normalized to the housekeeping/control gene (GAPDH).

Table 2.1 Genes included in TaqMan Low-Density array (TLDA) card.

Gene	Gene Name	Pathway (s)	Role
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Paclitaxel	Transporter
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	Carboplatin, paclitaxel	Transporter
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	Carboplatin, paclitaxel	Transporter
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	Carboplatin, paclitaxel	Transporter
ACTB	actin, beta	Control	House-keeping gene
ATP7A	ATPase, Cu++ transporting, alpha polypeptide	Carboplatin	Transporter
ATP7B	ATPase, Cu++ transporting, beta polypeptide	Carboplatin	Transporter
BIRC5	baculoviral IAP repeat-containing 5	Paclitaxel	Anti-apoptotic
BIVM/ERCC5	basic, immunoglobulin-like variable motif containing	Carboplatin	DNA repair
BRCA1	breast cancer 1, early onset	Carboplatin, paclitaxel	Genomic stability/Tumor suppressor
CCNB1	cyclin B1	Paclitaxel	Cell cycle regulator
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	Paclitaxel	Cell proliferation
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence)	Carboplatin	DNA repair
ERCC2	excision repair cross-complementing rodent repair deficiency, complementation group 2	Carboplatin	DNA repair
ERCC6	excision repair cross-complementing rodent repair deficiency, complementation group 6	Carboplatin	DNA repair
FOXC2	forkhead box C2 (MFH-1, mesenchyme forkhead 1)	Carboplatin, paclitaxel	Differetiation
FOXL1	forkhead box L1	Carboplatin, paclitaxel	Differetiation
GAPDH	glyceraldehyde-3-phosphate dehydrogenase-like 6; hypothetical protein LOC100133042; glyceraldehyde-3-phosphate dehydrogenase	Control	House-keeping gene
GSTM1	glutathione S-transferase mu 1	Carboplatin	Xenobiotic metabolism
GSTM3	glutathione S-transferase mu 3 (brain)	Carboplatin	Xenobiotic metabolism
GSTP1	glutathione S-transferase pi 1	Carboplatin	Xenobiotic metabolism
GSTT1	glutathione S-transferase theta 1	Carboplatin	Xenobiotic metabolism
HMGB1	high-mobility group box 1; high-mobility group box 1-like 10	Carboplatin	Pt-DNA adduct repair
KISS1	KiSS-1 metastasis-suppressor	Carboplatin	Metastasis suppressor *gene*
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	Paclitaxel	Microtubule- associated proteins
MAP2	microtubule-associated protein 2	Paclitaxel	Microtubule- associated proteins
MAP4	microtubule-associated protein 4	Paclitaxel	Microtubule- associated proteins

MAPT	microtubule-associated protein tau	Paclitaxel	Microtubule- associated proteins
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	Carboplatin	DNA repair
MPO	myeloperoxidase	Carboplatin	Xenobiotic metabolism
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	Carboplatin	DNA repair
MSH6	mutS homolog 6 (E. coli)	Carboplatin	DNA repair
NQO1	NAD(P)H dehydrogenase, quinone 1	Carboplatin	Xenobiotic metabolism
NR1I2	nuclear receptor subfamily 1, group I, member 2	Paclitaxel	Xenobiotic metabolism
PMS2	PMS2 postmeiotic segregation increased 2 (S. cerevisiae)	Carboplatin	DNA repair
POLB	polymerase (DNA directed), beta	Carboplatin	DNA repair
POLH	polymerase (DNA directed), eta	Carboplatin	DNA repair
REV3L	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)	Carboplatin	DNA repair
SLC22A7	solute carrier family 22 (organic anion transporter), member 7	Paclitaxel	Transporter
SLC31A1	solute carrier family 31 (copper transporters), member 1	Carboplatin	Transporter
SOD1	superoxide dismutase 1, soluble	Carboplatin	Xenobiotic metabolism
TP53	tumor protein p53	Carboplatin, paclitaxel	Tumor suppresor
XPA	xeroderma pigmentosum, complementation group A	Carboplatin	DNA repair
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1	Carboplatin	DNA repair
XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2	Carboplatin	DNA repair
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3	Carboplatin	DNA repair
XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining)	Carboplatin	DNA repair

2.2.9 Data analysis

All statistical analyses were performed using GraphPad Prism software version 7.0 and R statistical analysis software version 3.3.1, a free software environment as well as a programming language for statistical computing and graphics. Drug IC_{50} (effective dose that inhibits 50% of the cells), area under the survival curve (AUC), EC_{50} (concentration required to induce caspase 3/7 activity by 50%) and the area under the relative caspase activity curve (AUC) were also calculated using four parameter logistic dose response curves fitting to the *in vitro* drug response measurements (cell survival and caspase3/7 activity assays) [27].

To cluster cell lines based on cytotoxicity parameters, unsupervised K-means clustering was performed using the algorithm of Hartigan and Wong (1979) to identify the most-resistant and relatively sensitive subgroups of cell lines [30].

We used CalcuSyn software version 2.0 (Biosoft) based on Chou-Talalay method for the analysis of drug combination treatment [31]. This quantitative analysis of combination drug effects is based on the median-effect equation, derived from the mass-action law principle, that estimates the following: 1) combination index (CI) as the measure of the effect of each individual drug in combination treatment and 2) dose reduction index (DRI) or the reduction in dose requirement of a drug in combination treatment compare to single drug treatment and also 3) the required dose to achieve IC_{50} in combination (carboplatin + paclitaxel) treatment for each cell line [31]. Combination index (CI) is the quantitative measure of interactions between multiple drug effects interactions derived from the median-effect equation or Unified theory of the law of Mass-Action, where $CI<I$ is defined as synergistic, $CI>1$ as antagonistic and $CI=1$ as additive [31]. Additionally, DRI or dose reduction index (DRI) of an individual drug in combination treatment is a measure of estimated reduction of its dose at a given effect level compared to when the drug is administered as a single agent. Therefore, higher DRI corresponds to lower dose requirement which implies reduced toxicity at the increased effect in synergistic combination [31].

The association of SNPs with *in vitro* drug response phenotypes IC_{50} or EC_{50} was evaluated with linear models. For annotation of results across gene regions, SNPs were mapped to genes within 2KB using Biofilter (assembly CRCh37.p10, genome build 104.0) and SNPNexus, a web-based application for the selection of functionally relevant Single Nucleotide Polymorphisms (SNP) for large-scale genotyping studies [27].

Gene expression data generated using TaqMan Low Density arrays (TLDA) was analyzed using Partek Genomics Suite software v6.6 to perform differential expression analysis and to find correlation between drug pathway gene expression signatures with drug chemo-sensitivity parameters using Analysis of variance (ANOVA) model. Heatmaps were generated for each set of pathway genes (Carboplatin and paclitaxel) using unsupervised hierarchical clustering (HC) analysis based on the genes expressed differentially. Gene expression data was standardized: shifted to mean=0 and scaled to standard deviation=1 prior to Hierarchical clustering analysis.

2.3 Results

2.3.1 *In vitro* profiling of carboplatin and paclitaxel chemo-sensitivity

A total of 112 EBV-transformed ovarian cancer cell lines were created. Among these, 81 cell lines were successfully treated with carboplatin and paclitaxel as single agents and as combination. **Figure 2.1** demonstrates the quality control (QC) steps undertaken to check for variability or batch effects in the data owing to variations of experimental conditions. Briefly, a few cell lines were randomly selected and cytotoxicity assays were performed at multiple time points to check for consistency. Our results showed there were no batch effects observed for our drug cytotoxicity or caspase activity data. Furthermore, we divided the dataset into two subsets and performed statistical verification of any batch effect, as previously reported [27].

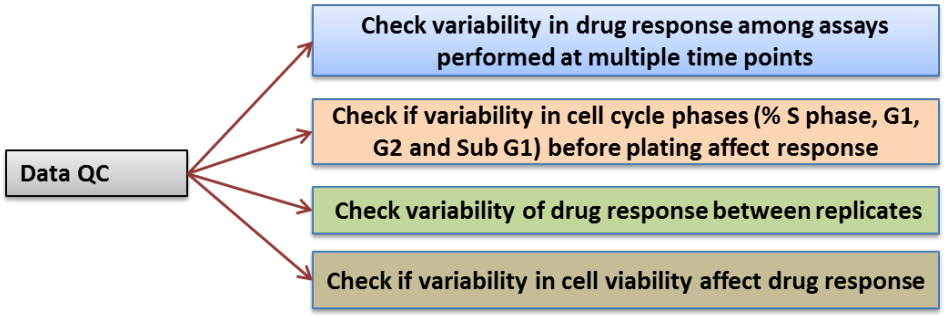


Figure 2.1 Workflow representing Data QC (quality check) of chemo-sensitivity assay results

Table 2.2 provides a summary of all the *in vitro* chemo-sensitivity measures assessed for single agent and combination treatments, including IC_{50}, AUC, Caspase AUC and Caspase EC_{50}. Results from our *in vitro* chemo-sensitivity analysis showed wide variability in response to carboplatin and paclitaxel treatment as single agent among LCLs from ovarian cancer patients. For carboplatin cytotoxicity, IC_{50} and AUC showed fold changes of 8.34 and 3.84 between the lowest (most-sensitive) and highest (most-resistant) values, respectively whereas the fold changes were 7.72 and 5.65, respectively for paclitaxel**.**

Boxplots in **Figure 2.2** represent the distribution of log transformed drug-sensitivity parameters across the LCL panel. **Figure 2.3** depicts a scatterplot matrix showing the correlation between the

observed drug chemo-sensitivity parameters in EOC-LCLs. We observed a significantly high ($r>0.5$; $p<0.0001$) correlation between the following: caspase AUC values for combination treatment with caspase AUC for carboplatin (r= 0.86) and paclitaxel (r= 0.84) as single agent treatment; paclitaxel IC50 vs paclitaxel AUC (r=0.71); paclitaxel IC50 vs Combination AUC (r=0.59); Combination AUC vs paclitaxel AUC (r=0.7); Combination AUC vs carboplatin AUC (r=0.59); and carboplatin caspase vs paclitaxel caspase (r=0.74) (**Figure 2.3**). As expected, IC_{50} and AUC values were negatively correlated with corresponding caspase AUC values for a treatment regimen indicating increase in caspase 3/7 activity and apoptosis in the dying cells.

Our results demonstrate a wide range of diversity in response to treatment with carboplatin and paclitaxel single agent treatment as well as strong correlation with combination treatment. Our caspase 3/7 activity data corroborated with cytotoxicity data (data not shown).

We also performed flow cytometry analysis of the cell lines prior to performing chemosensitivity assays to assess cell cycle phases based on quantification of DNA content. Our results showed no correlation between cell cycle phases and drug cytotoxicity revealing drug response was not influenced by either cell viability or cell cycle phases **(Table 2.3).**

Table 2.2 Summary of *in vitro* phenotype measures in LCLs from EOC patients.

Drug	Phenotype	N	Mean	Minimum	Maximum	Fold Change (Max/Min)
Carboplatin	IC_{50} (μM)	77	52.56	13.63	113.24	8.31
Carboplatin	AUC	81	6165.32	2759	10598	3.84
Carboplatin	Caspase_AUC	79	288.23	100	640	6.4
Carboplatin	Caspase_EC_{50} (μM)	79	27.12	6	96	16
Paclitaxel	IC_{50} (nM)	76	19.33	5.84	45.12	7.72
Paclitaxel	AUC	81	3159.52	1165	6584	5.65
Paclitaxel	Caspase_AUC	79	173.49	80	330	4.13
Paclitaxel	Caspase_EC_{50} (μM)	79	10.75	2.9	56	19.31
Combination	AUC	81	2917.99	1262	6568	5.2
Combination	Caspase_AUC	79	139.35	62	270	4.35
Combination	Caspase_EC_{50}	79	10.73	3.7	41	11.08

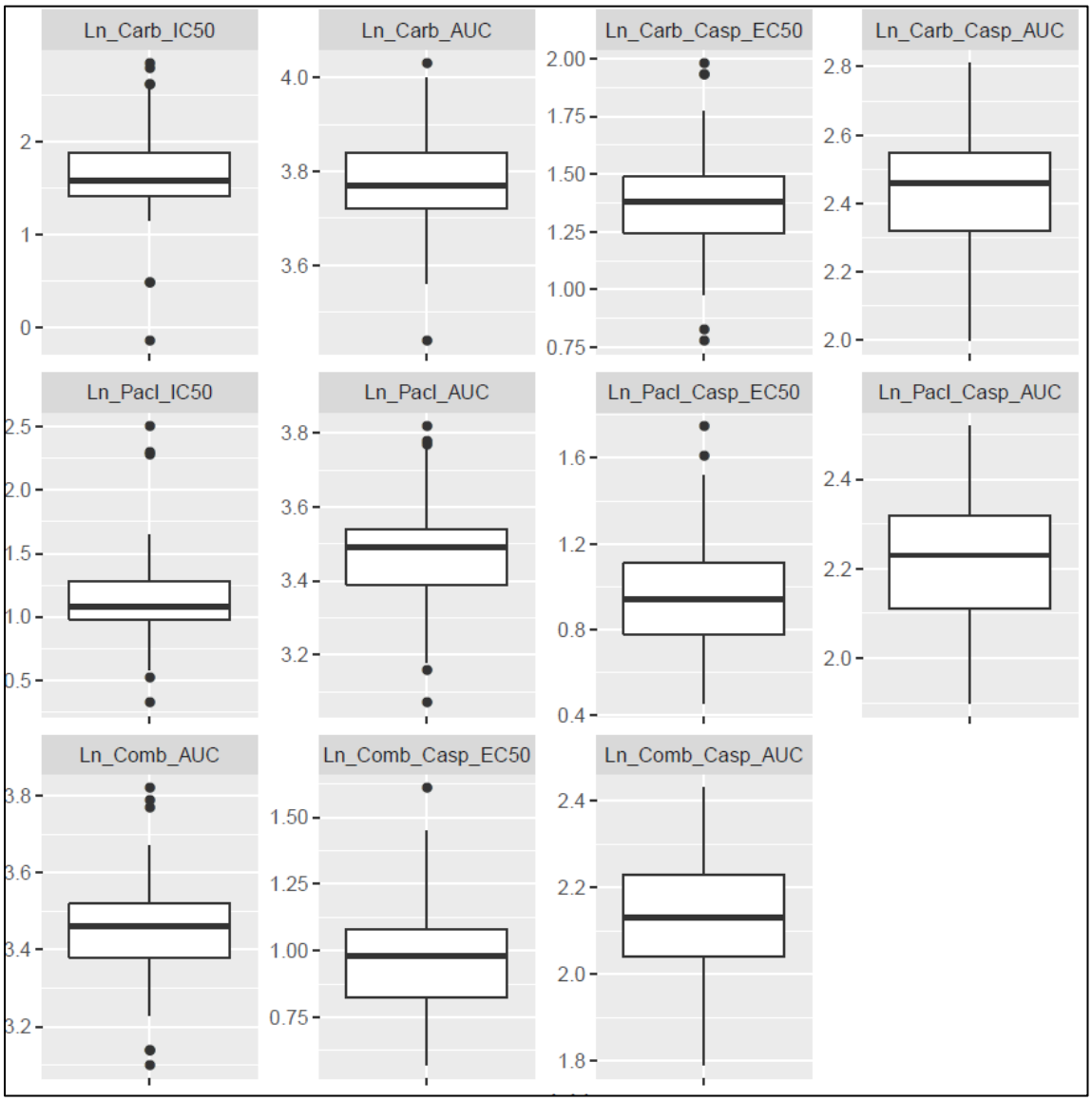


Figure 2.2 Boxplots representing distribution of a) drug cytotoxicity and b) caspase activity following treatment.

Cytotoxicity values were log-transformed (represented in the y-axes) and boxplots were generated using the R statistical packages. (Carb = Carboplatin; Pacl = Paclitaxel; Comb = Combination; Casp = Caspase)

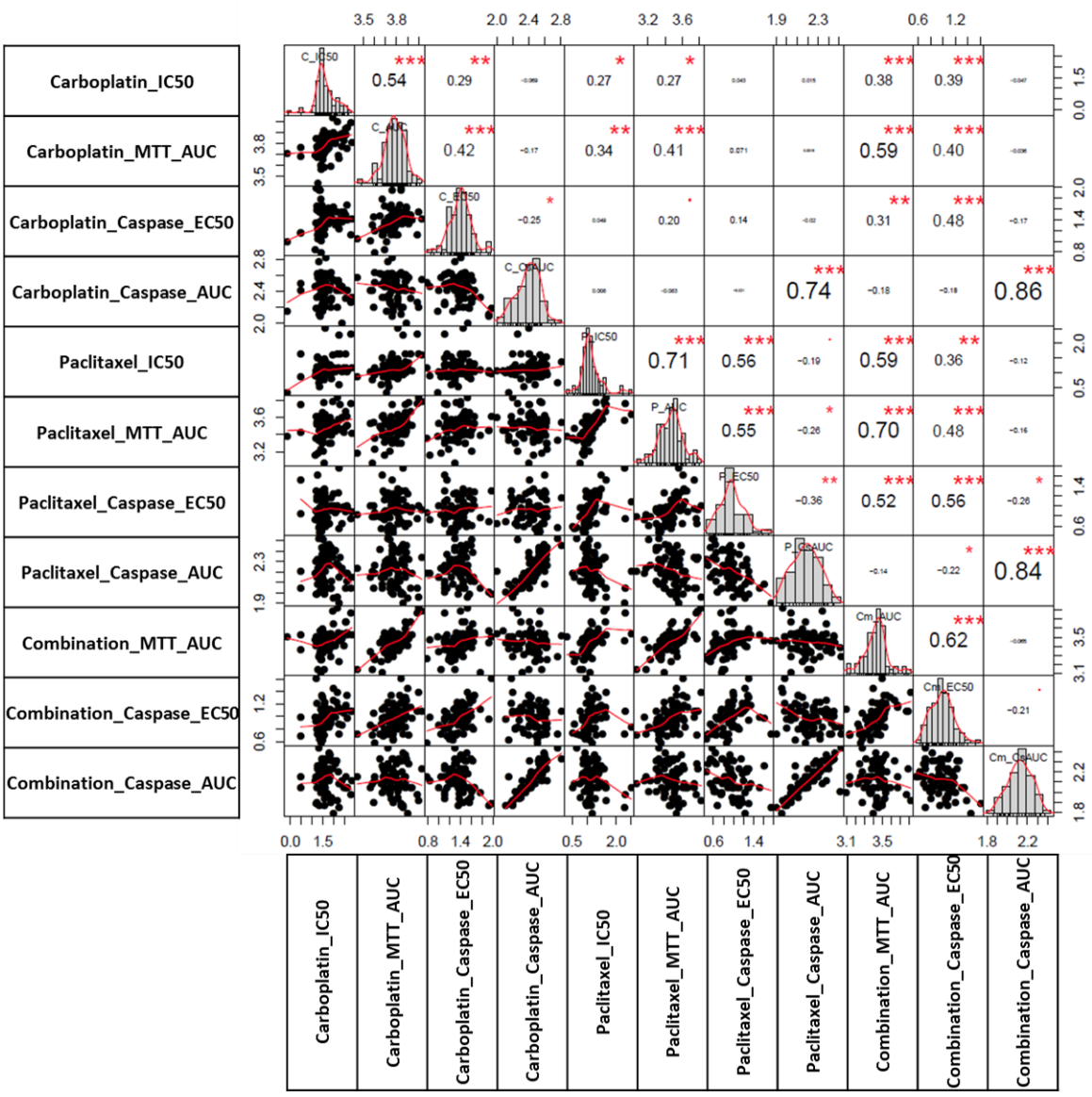


Figure 2.3 Scatterplot matrix representing pairwise correlations between IC_{50} and AUC estimates of paclitaxel and carboplatin single-agent and combination treatment.
Cytotoxicity estimates were log-transformed and a scatterplot matrix was generated using the R graphing package. Scatterplots, r values and corresponding significance (pvalues) for each pair-wise correlation are included. Marginal plots on the diagonal axis represent histograms of the individual cytotoxicity parameters. Significance levels are associated with the following symbols and represent p-values (0.001, 0.01, 0.05, 0.1, 1) <=> symbols (“***”, “**”, “*”, “.”, " “)

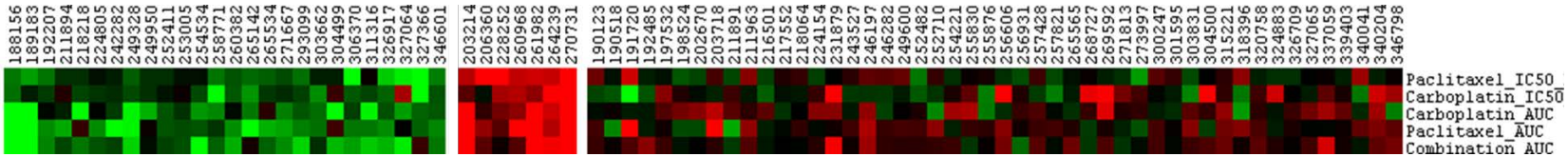
Table 2.3 Summary of correlation between cell viability & cell cycle phases and drug IC_{50}

Phenotype	Minimum	Mean	Maximum	sd	Carboplatin. IC_{50}	Paclitaxel. IC_{50}	Combination. IC_{50}
Cell viability %	80	91.33	97	3.81	r= 0.2156	r = 0.1135	r= 0.1375
S-Phase %	12.3	24.843	38.36	4.997	r=0.042	r=-0.1049	r=-0.099
G1- Phase %	41.62	63.072	76.35	6.906	r=-0.126	r=-0.142	r=-0.165
G2- Phase %	1.01	11.007	34.95	5.654	r=0.051	r=0.254	r=0.218

2.3.2 K-Means clustering identified resistant subgroups

K-Means clustering analysis was performed to identify clusters or subgroups based on our based on our *in vitro* cytotoxicity parameters observed in the EOC-LCLs [30]. Three (3) distinctly different drug response subgroups were identified (**Figure 2.4**). We then focused on identifying differences in response to carboplatin, paclitaxel and combination treatments between the 3 subgroups generated using K-Means clustering. **Table 2.4** provides numerical summary of the drug response parameters for each K-means cluster. Our results showed that cytotoxicity AUC and IC_{50} for carboplatin, paclitaxel and combination were significantly higher ($p< 0.0001$) in Cluster 2 (resistant subgroup) compared to other cell lines. 7 cell lines (Cluster 2) were identified as most resistant to the drug treatments. Cluster1 (n= 26) was identified as the most sensitive while cluster 3 (n=84) is the intermediate subgroup **(Figure 2.4)**. Pvalues of Kruskal-Wallis test by ranks are also provided for each drug response phenotype (**Figure 2.5**).

a)



b)

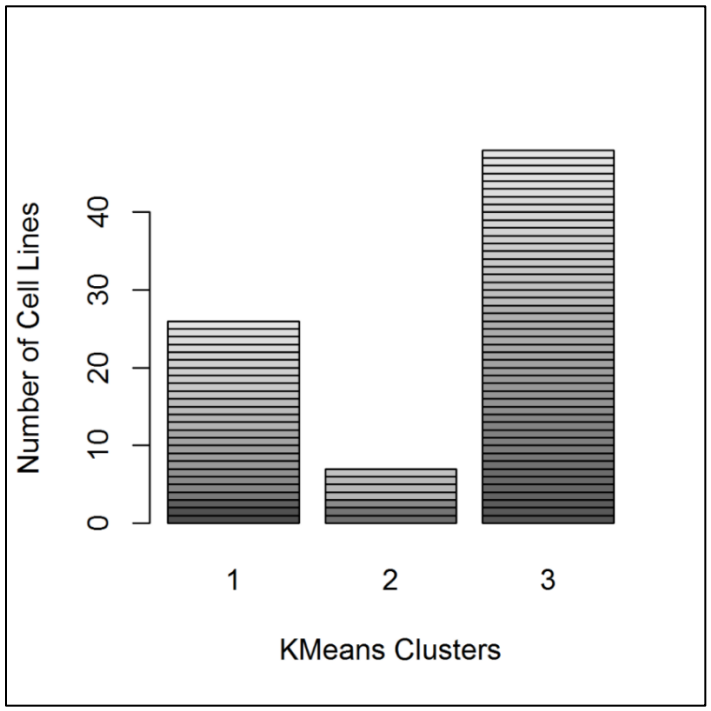


Figure 2.4 Results from Kmeans clustering of *in vitro* cytotoxicity data

a) Heatmap representing Kmeans clusters generated using *in vitro* cytotoxicity assay data on the LCL panel. K Means clustering (k=3) identified 7 cell lines (middle panel) as most resistant to the drug treatments; b) Bar plots showing subgroups of cell lines in the LCL panel clustered using Kmeans clustering analysis (k=3)

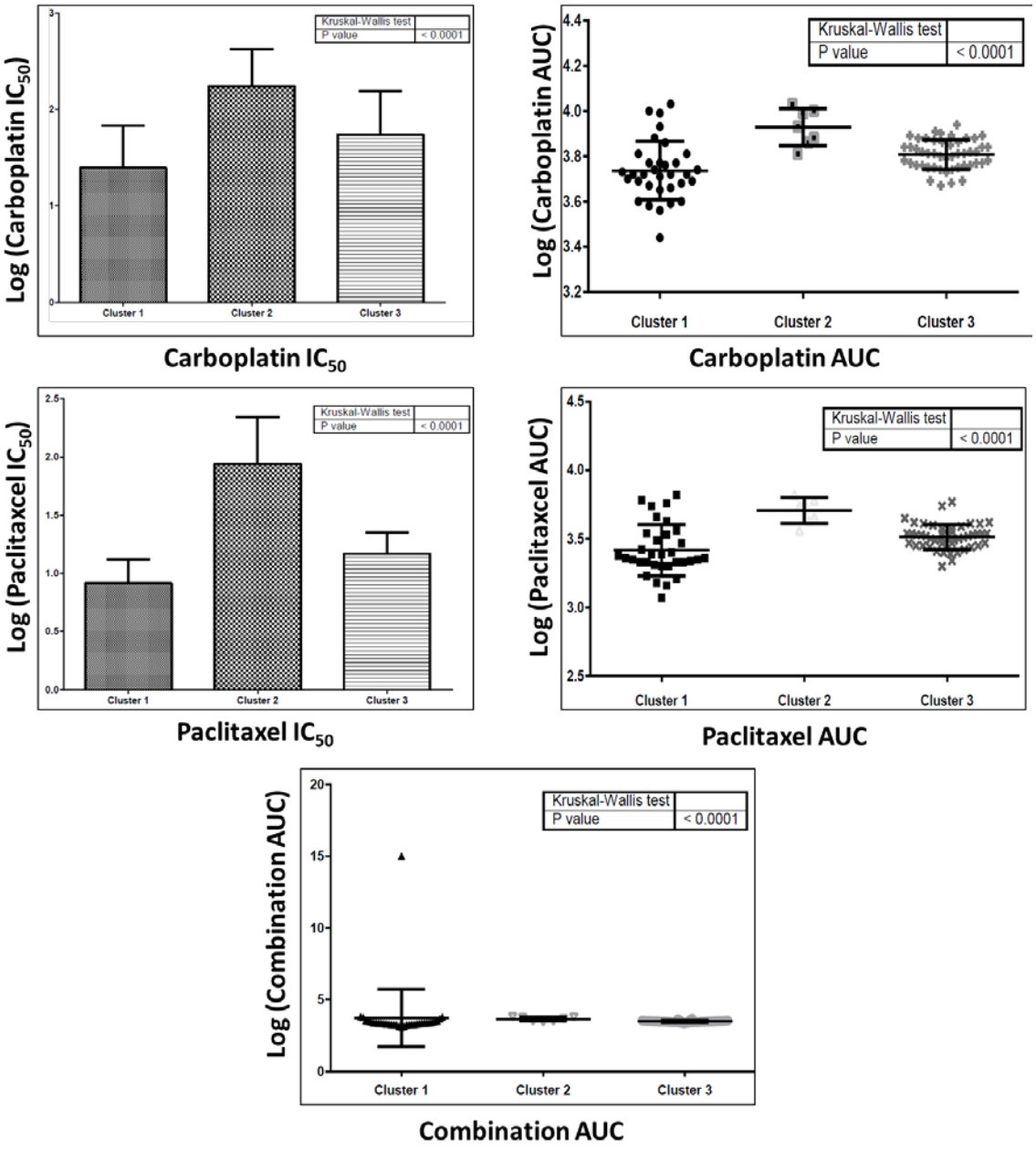


Figure 2.5 Plots showing differences in response to carboplatin, paclitaxel and combination treatments between the clusters generated using in vitro cytotoxicity assay data on the LCL panel.

K-Means clustering analysis was performed to identify clusters or subgroups based on our based on our *in vitro* cytotoxicity profiling data in the cell lines.

Table 2.4 Summary of drug response phenotypes in subgroups identified by Kmeans clustering.

Drug	Phenotype	Cluster 1 (n=26)	Cluster 2 (n=7)	Cluster 3 (n=48)	P (Kruskal-Wallis)
Carboplatin	IC_{50} (μM)	34.99	236.43	99.69	< 0.0001
Paclitaxel	IC_{50} (nM)	8.98	125.57	16.12	< 0.0001
Carboplatin	AUC	4907.73	8580.43	6494.31	< 0.0001
Combination	AUC	2120.5	4706	3089.21	< 0.0001
Paclitaxel	AUC	2255.88	5191.43	3352.67	< 0.0001

2.3.3 Analysis of carboplatin-paclitaxel combination treatment using CI theorem

Furthermore, we investigated the *in vitro* interactions between the two drugs, carboplatin and paclitaxel, in combination compared to use as single agent in our cell line panel of 81 EOC-LCLs using Chou-Talalay's Combination Index-Isobologram Theorem to evaluate synergism, antagonism or additive effects [31]. We studied the effect of combination (carboplatin + paclitaxel) treatment in our cell line panel of 81 EOC-LCLs. Results showed, for either of the drugs, reduced dose was required to achieve a similar effect (% kill) in combination treatment compared to single agent treatment for majority of the cell lines. We also observed large variability in the IC_{50} of single-agent treatment compared to its IC_{50} when used in drug combination (**Figure 2.6**). Our observed CI values showed wide variation (0.14-5.51) within the cell line panel. As mentioned in the Methods section, where CI<I is defined as synergistic, CI>1 as antagonistic and CI=1 as additive [31]. We also observed that dose reduction (DRI) for a drug is low in cell lines where the combination results in predicted antagonistic drug-drug interaction (high CI value). High DRI corresponds to lower dose requirement, as described earlier, which suggests reduced toxicity corresponding to increasing synergism in combination treatments [31].We also observed high negative correlation between DRI and CI for carboplatin IC_{50} (r=- 0.732), as well as for paclitaxel IC_{50} (r=-0.641). **Figure 2.7** summarizes the CI and DRI values in the diagonal box plots and provides pair-wise correlations between CI and DRI values at predicted IC_{25}, IC_{50} and IC_{75}. Thus, our DRI calculations revealed lower dose requirements to achieve similar anti-cancer effects when

the drugs were used in combination, compared to single-agent regimens, based on our observations from their reduced predicted IC_{50} and IC_{75} values in combination treatments (**Table 2.5**).

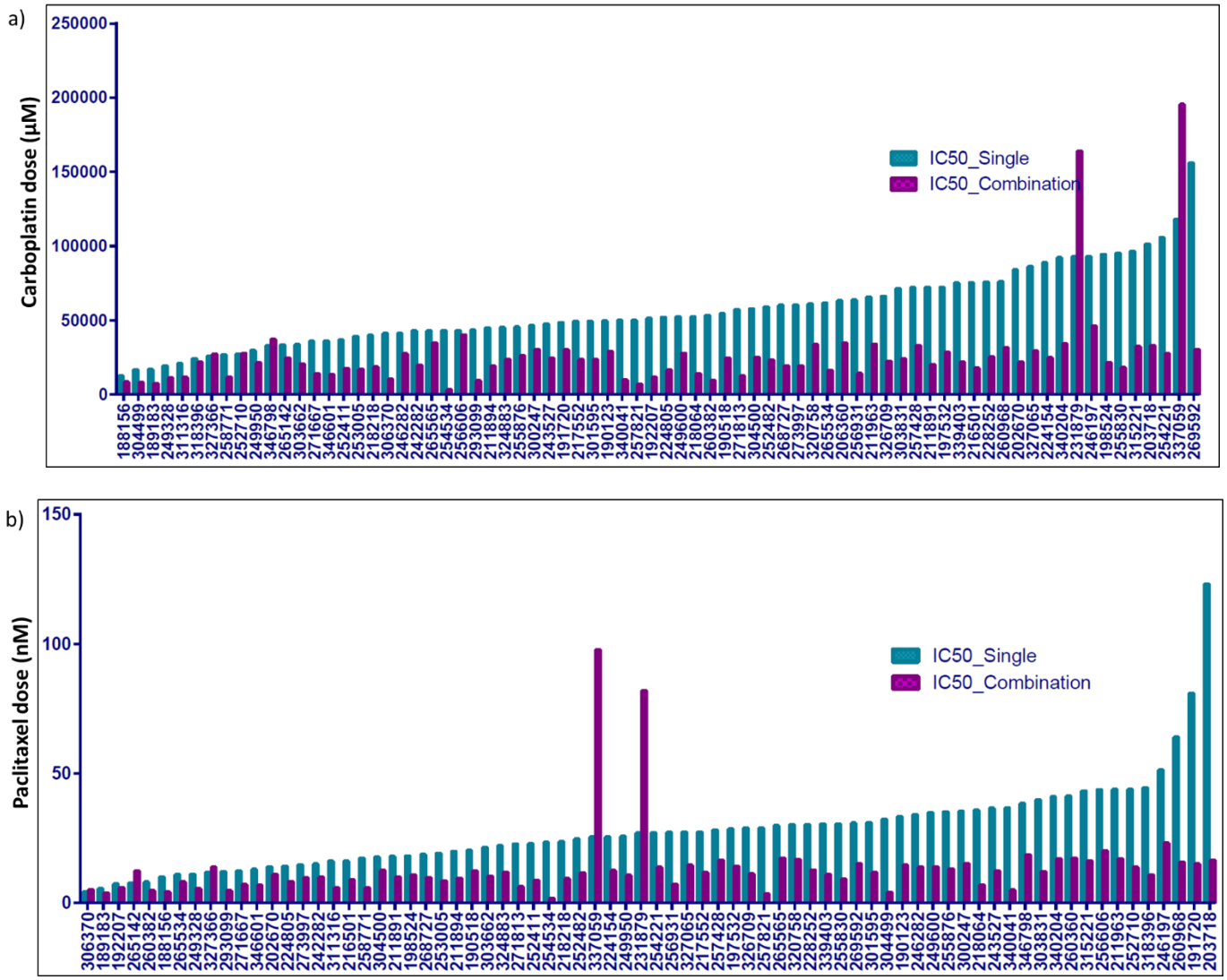


Figure 2.6 Representation of variation of the relative IC_{50}s of treatment with single agents and drug combination that indicates variation across LCL panel. a) carboplatin; b) paclitaxel.

Table 2.5 CI and DRI as measures of Synergism and Antagonism of drug combination treatment in LCLs from EOC patients.

	Drug Phenotype	Minimum	Mean	Maximum
CI	IC_{50}	0.14	1.03	5.51
DRI	Carboplatin IC_{50}	0.57	2.84	14.04
DRI	Carboplatin IC_{75}	0.00	2.88	11.23
DRI	Paclitaxel IC_{50}	0.26	2.74	15.26
DRI	Paclitaxel IC_{75}	0.00	2.14	7.79

Combination Index (CI) and Dose-Reduction Index (DRI) values were calculated using Compusyn software program that applies Median-Effect Principle (by Chou) and the Combination Index-Isobologram Theorem (by Chou-Talalay). CI < 1, Synergism; CI = 1, Additive; CI > 1, Antagonism.

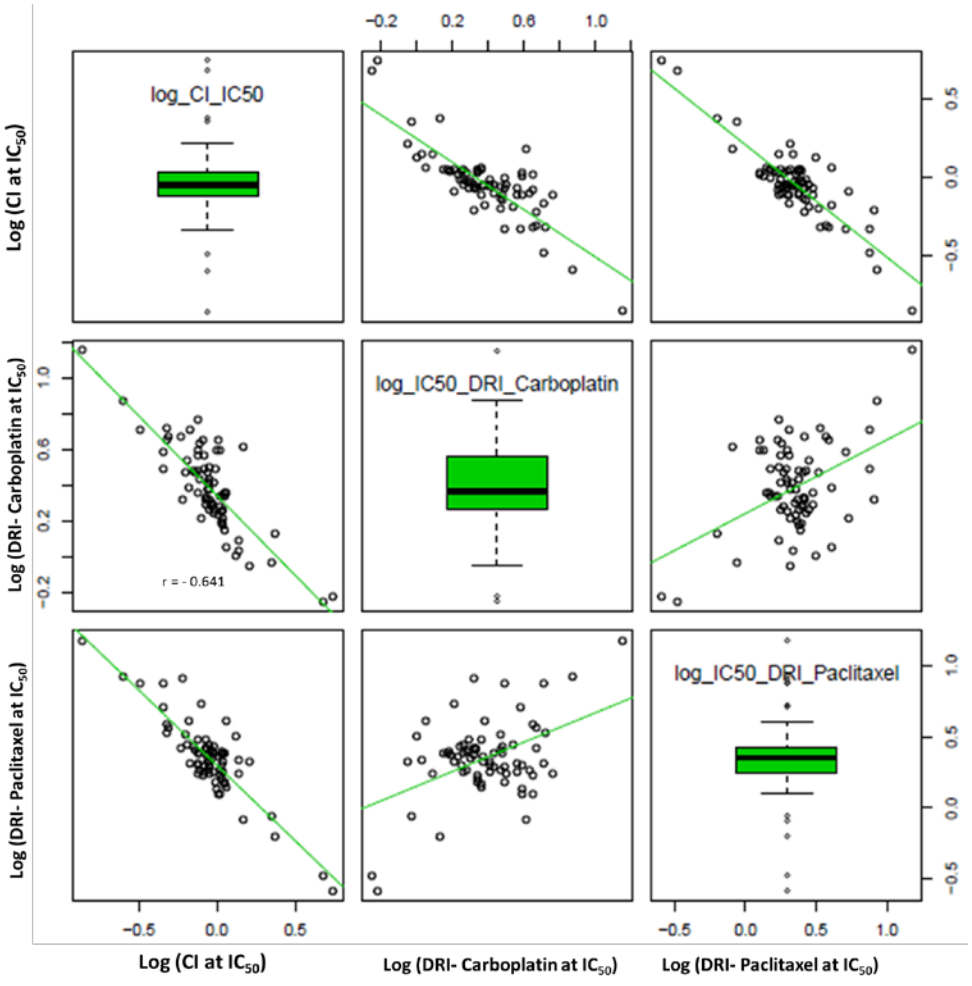


Figure 2.7 Scatterplots showing correlation between combination index (CI) and dose reduction index (DRI) values at IC_{50}.
Diagonal axis shows boxplots of the distribution of each parameter.

2.3.4 SNPs in pathway genes associated with chemo-sensitivity

Genotype call rates observed for all samples were >95%, and minor allele frequency (MAF) ≥0.01. We performed a genotype-phenotype association analysis between SNPs in carboplatin and platinum pharmacokinetic and pharmacodynamics pathway genes extracted from our GWAS study and our observed *in vitro* chemo-sensitivity measures. **Table 2.6** provides details of the top 15 pathway SNPs that were found significantly associated with *in vitro* cytotoxicity phenotypes and their predicted functional effects. We identified significant association between pathway genes of drugs with response to carboplatin, paclitaxel as single agent and as combination therapy. At $p<0.01$, 85 carboplatin pathway gene mutations were significantly associated with carboplatin IC_{50}, 50 paclitaxel pathway genes were associated with paclitaxel IC_{50} while a total of 67 genetic variations were significantly associated with combination treatment chemosensitivity.

Analysis of association between genetic variations in drug PK/PD pathway genes and *in vitro* caspase activity revealed 46, 23, 63 mutations associated with caspase activity in carboplatin single agent, paclitaxel single agent and combination treatment, respectively. **Table 2.7** lists the top 15 Pathway SNPs that were found most significantly associated with caspase activity phenotypes and their predicted functional effects.

2.3.4.1 SNPs in genes associated with drug pharmacokinetics

Top among the SNPs in drug disposition (PK) genes significantly ($p<0.05$) associated with carboplatin cytotoxicity were the SNPs rs186868889 (p=0.001) and rs72659636 (p=0.001) in the gene *ABCG2* and rs182123265 (*ABCC2*; p=0.002). Most significant SNP versus paclitaxel single-agent cytotoxicity association was found among rs1153867 (*ABCC1*) and rs9472030 (p=0.002). On the other hand, cytotoxicity of carboplatin-paclitaxel combination therapy was highly significantly associated with the following SNPs: rs183572778, rs17224787, rs62140866, rs17224528 (*MSH2*); rs3887412 (p=0.0034), rs35598 (p=0.0035), rs35599 (p=0.0035) (*ABCC1*). The top PK pathway gene mutations associated with caspase activity following carboplatin

treatment include the transporter genes *ATP7B* and the xenobiotic metabolism gene *GSTM1*. Mutations in the genes *MAP4*, *MAPT*, *MAP2*, were among the top caspase activity-associated PK gene variations following paclitaxel single-agent treatment. Finally, genetic variations in *ABCC1*, *BIRC5*, *MAP4*, and *GSTP1* were highly associated with caspase activity following treatment with carboplatin and paclitaxel as combination.

2.3.4.2 SNPs in genes related to pharmacodynamics response

The following SNPs in pharmacodynamics (PD) genes were most significantly associated with carboplatin cytotoxicity: rs1425118 (p=0.003), rs55911615 (p=0.003), rs6749415 (p=0.003) in *XRCC5*, and rs75475960 (*MSH2*; p=0.003). On the other hand, significantly high SNP vs paclitaxel single-agent cytotoxicity association was found among *TP53* SNPs rs17887200, rs17881556 and rs17886760 (p<0.001 for all three SNPs), and the *EGFR* SNPs rs7779645, rs7804688, rs6947594, rs6593209 (p<or =0.001 for all three SNPs), The top PD pathway gene mutations associated with caspase activity following carboplatin treatment include DNA repair genes *XRCC5*, *XRCC1*, *ERCC2*, and *ERCC1*, as well as *REV3L*, *MSH6* and *KISS1*. Top PD pathway gene mutations associated with caspase activity following carboplatin treatment include: mutations in the genes *BIVM/ERCC6, EGFR* and *BRCA1* were among the top caspase activity-associated PD variations following paclitaxel single-agent treatment. Whereas, genetic variations in *BIRC5*, *KISS1*, and *EGFR* were highly associated with caspase activity following treatment with carboplatin and paclitaxel as combination.

Table 2.6 Top pathway SNPs found most significantly associated with *in vitro* cytotoxicity parameters.

	Chr	chromPosition	dbSNP ID	*Gene/Gene*	Allele 1	Allele 2	Direction	P-value (SNP vs IC50)	Predicted function
Carboplatin IC50									
	2	217037273		*XRCC5*	t	c	-1	0.001	
	4	88994224	rs186868889	*ABCG2*	t	g	-1	0.001	
	4	88999267	rs72659636	*ABCG2/PKD2*	c	g	-1	0.001	3downstream
	7	6061988		*PMS2/EIF2AK1*	a	g	1	0.001	3utr
	2	217028174		*XRCC5*	a	g	-1	0.002	
	10	101573872	rs182123265	*ABCC2*	a	g	1	0.002	
	2	216958899	rs1425118	*XRCC5*	c	g	1	0.003	
	2	217028646		*XRCC5*	t	g	-1	0.003	
	2	216958412		*XRCC5*	a	g	-1	0.003	
	2	216958402	rs6749415	*XRCC5*	a	g	1	0.003	
	2	216968340	rs55911615	*XRCC5/TMEM169*	a	g	1	0.003	3downstream
	2	47616643	rs75475960	*MSH2*	c	g	1	0.003	
	2	216970326		*XRCC5*	t	c	-1	0.003	
	2	216972934		*XRCC5/XRCC5*	a	g	1	0.003	5upstream
Paclitaxel IC_{50}									
	17	7571071	rs17887200	*TP53*	t	c	-1	<0.001	3downstream
	17	7567681		*TP53*	t	c	1	<0.001	
	17	7568628		*TP53*	a	g	1	<0.001	
	7	55185302	rs7779645	*EGFR*	a	t	-1	<0.001	
	7	55190098	rs7804688	*EGFR*	a	t	-1	<0.001	

	7	55203297	rs6947594	*EGFR*	a	c	-1	<0.001	
	7	55203838		*EGFR*	a	g	1	<0.001	
	17	7570869	rs17881556	*TP53*	t	c	-1	0.001	3downstream
	17	7570978	rs17886760	*TP53*	t	c	-1	0.001	3downstream
	17	7570823		*TP53*	c	g	1	0.001	3downstream
	17	7570956		*TP53*	a	g	1	0.001	3downstream
	16	16203202	rs1153867	*ABCC1*	t	g	1	0.001	
	7	55200625	rs6593209	*EGFR*	a	g	-1	0.001	
	6	43271237	rs9472030	*SLC22A7/CRIP3*	t	g	-1	0.002	3downstream
	7	55208284		*EGFR*	a	c	1	0.002	
Combination									
	2	48010163		*MSH6*	t	c	1	0.0007	5upstream
	2	47636927	rs183572778	*MSH2*	a	g	-1	0.0011	
	16	16173221		*ABCC1*	t	c	-1	0.0012	S667S
	2	47701362	rs17224787	*MSH2*	a	t	1	0.0019	
	2	47682634	rs62140866	*MSH2*	a	c	1	0.0021	
	2	47684043	rs17224528	*MSH2*	a	g	1	0.0022	
	16	16173516		*ABCC1*	t	c	-1	0.0034	
	16	16175030	rs3887412	*ABCC1*	a	t	1	0.0034	
	16	16182528		*ABCC1*	t	c	-1	0.0034	
	16	16183045		*ABCC1*	a	g	-1	0.0034	
	16	16159023	rs35598	*ABCC1*	a	g	1	0.0035	
	16	16159061	rs35599	*ABCC1*	t	c	1	0.0035	
	16	16168608		*ABCC1*	t	c	-1	0.0039	
	16	16169465		*ABCC1*	t	g	-1	0.0039	
	16	16170771		*ABCC1*	a	g	-1	0.0039	

Table 2.7 Top pathway SNPs found significantly associated with *in vitro* combination treatment caspase activity.

	Chr	chromPosition	dbSNP ID	Gene	Allele1	Allele2	Direction	P-value (SNP vs Caspase)	Predicted function
Carboplatin									
	1	110211681	rs428135	*GSTM1*	C	g	1	0.001	
	2	217003152	rs2303400	*XRCC5*	T	c	1	0.002	
	6	111801193		*REV3L*	A	g	1	0.003	
	13	52497540		*ATP7B*	A	g	-1	0.003	
	19	44062781		*XRCC1*	D	r	-1	0.003	
	19	45837542		*ERCC2*	C	g	1	0.003	
	19	45841318		*ERCC2*	A	g	1	0.003	
	2	217012092	rs2160981	*XRCC5*	A	g	1	0.004	
	19	44041363		*XRCC1*	T	c	-1	0.004	3’ downstream
	19	45931387	rs28586606	*ERCC1*	A	g	1	0.004	
	19	45936292		*ERCC1*	T	c	-1	0.004	
	1	110291406		*GSTM3*	T	c	-1	0.005	3’ downstream
	1	204181317	rs72749759	*KISS1*	A	c	-1	0.005	
	2	48012375		*MSH6*	C	g	-1	0.005	
	2	48022688	rs111861797	*MSH6*	T	c	1	0.005	
Paclitaxel									
	10	96845842		*CYP2C8*	D	r	-1	0.003	
	3	47977447		*MAP4*	D	r	1	0.005	
	17	44072651		*MAPT*	A	g	1	0.005	
	17	44090778		*MAPT*	A	t	1	0.007	
	17	44090779		*MAPT*	A	c	1	0.007	
	2	210512893		*MAP2*	A	g	-1	0.008	
	2	210574383		*MAP2*	A	g	1	0.008	
	2	210596950	rs114531804	*MAP2*	A	g	1	0.008	3’ UTR
	7	55197234	rs79884022	*EGFR*	T	c	-1	0.008	

	7	55206022		*EGFR*	A	g	1	0.008	
	17	41250221		*BRCA1*	D	r	-1	0.008	
	17	41260985		*BRCA1*	C	g	-1	0.008	
	2	210534297	rs141068360	*MAP2*	T	g	-1	0.009	
	3	47896738		*MAP4*	A	c	1	0.009	Coding (K1087N)
	3	47918295		*MAP4*	A	g	1	0.009	
Combination									
	17	76238765		*BIRC5*	T	c	-1	<0.001	3’ downstream
	17	76239023	rs7223704	*BIRC5*	T	c	1	<0.001	3’ downstream
	17	76231842		*BIRC5*	T	c	-1	0.002	
	3	48067426	rs184824121	*MAP4*	T	c	-1	0.003	
	1	204150976		*KISS1*	T	c	1	0.005	
	7	55124701	rs6593202	*EGFR*	T	c	-1	0.005	
	11	67362385	rs7108038	*GSTP1*	A	g	-1	0.005	
	16	16195257		*ABCC1*	A	g	1	0.005	
	16	16206501	rs45499397	*ABCC1*	C	g	-1	0.005	
	17	76230981	rs8064778	*BIRC5*	C	g	1	0.005	
	17	76231434		*BIRC5*	A	t	-1	0.005	
	11	67358076		*GSTP1*	T	g	1	0.006	
	11	67359090	rs7939505	*GSTP1*	C	g	-1	0.006	
	11	67359718	rs6591259	*GSTP1*	A	g	-1	0.006	
	11	67359947	rs7937159	*GSTP1*	T	g	-1	0.006	

2.3.5 Differential GEP signatures of drug response

By comparing gene expression versus response phenotype using the Spearman rank-order correlation analysis, several key pathway genes were found significantly associated with drug chemosensitivity phenotype measure **(**details provided in **Table 2.8)**.

Pre-treatment gene expression levels of candidate genes involved in carboplatin and paclitaxel PK/PD pathway were assayed using TaqMan Low Density Arrays. Average Ct values for each gene were then normalized using average Ct value of the housekeeping/control gene (GAPDH). Data was log-transformed and used for analysis of association with phenotype measures. Genes showing no expression in >5% cell lines were filtered out prior to analysis. Differential gene expression analysis was performed using the remaining genes to identify gene expression changes that were highly correlated with drug chemo-sensitivity. Clustering analysis using the most differentially expressed genes revealed distinct clusters/subgroups within the LCL panel. **Figure 2.8 and 2.9** show heatmaps that cluster the EOC LCL panel based on most differentially expressed pathway genes for carboplatin and paclitaxel, respectively.

Expression of the following genes were highly correlated with carboplatin cytotoxicity: *ERCC2* (p=0.015); *GSTM1* (p=0.0097); *GSTT1* (p=0.0086); *NQO1* (p=0.0497), while expression levels of the following genes were correlated with response to paclitaxel single agent treatment: *BIRC5* (p=003); *FOXL1* (p=0.0148); *FOXC2* (p=0.0034). When compared to the phenotype measures of carboplatin-Paclitaxel combination treatment, CI values at IC_{50} was correlated with the carboplatin pathway genes *ATP7A* and *ATP7B*. In addition, DRI values at IC25 were correlated with the following carboplatin pathway genes: *POLH* (p=0.022); *ATP7A* (p=0.042) and *FOXC2* (p=0.032), as well as the paclitaxel pathway genes *FOXC2* (p=0.0114), *BIRC5* (p=0.0237) and *EGFR* (p=0.0458).

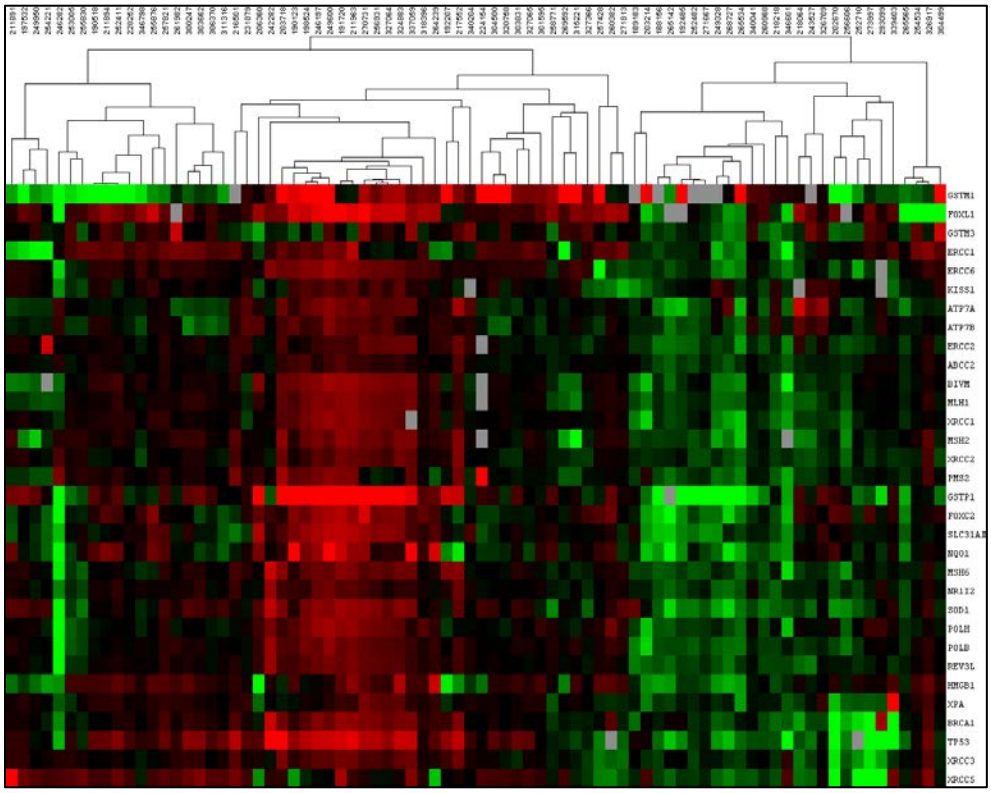
Spearman correlation analysis between caspase activity and pathway gene expression revealed trends towards significance for expression of the genes *GSTP1* (p=0.08) and *NQO1*(p=0.096) for

carboplatin treatment and *FOLXL1* (p=0.07), *BIRC5* (p=0.07) and *EGFR* (p=0.08) for caspase activity induced by paclitaxel single agent treatment (**Table 2.8**).

Table 2.8 Summary of results from Spearman correlation analysis between TLDA gene expression and drug chemo-sensitivity outcomes (p<0.1).

Drug	Phenotype	Column ID	p-value (correlation)
Carboplatin	IC50	*ERCC2*	0.035
		XPA	0.087
	MTT.AUC	*GSTM1*	0.010
		ERCC2	0.002
		MSH2	0.040
		TP53	0.083
		GSTP1	0.023
		NQO1	0.032
		MSH6	0.035
		XRCC2	0.037
		SOD1	0.039
		ATP7A	0.039
		POLB	0.043
		FOXL1	0.045
		REV3L	0.082
		XRCC3	0.096
	Caspase.AUC	*GSTP1*	0.085
	Caspase.EC50	*NQO1*	0.096
Paclitaxel	IC50	*BIRC5*	0.094
	MTT.AUC	*BIRC5*	0.002
		FOXC2	0.003
		FOXL1	0.011
		MAD2L1	0.093
	Caspase.EC50	*FOXL1*	0.070
		BIRC5	0.074
Combination	MTT.AUC	*GSTM1*	0.017
		ERCC6	0.053
		BIRC5	0.039
		FOXC2	0.064
		NQO1	0.085
		FOXL1	0.090
	Casp.AUC	*GSTP1*	0.022
	Casp.EC50	*XPA*	0.017
		HMGB1	0.029
		BIRC5	0.050
		ERCC1	0.061
		NQO1	0.064
		FOXC2	0.078

a)



b)

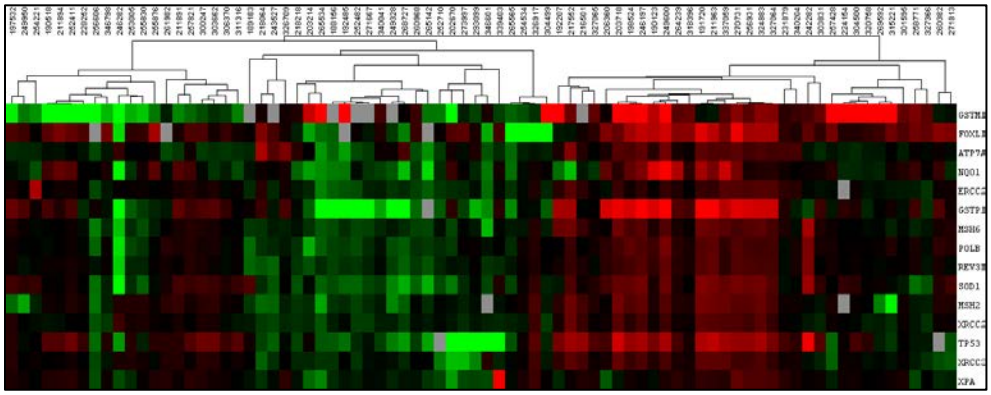
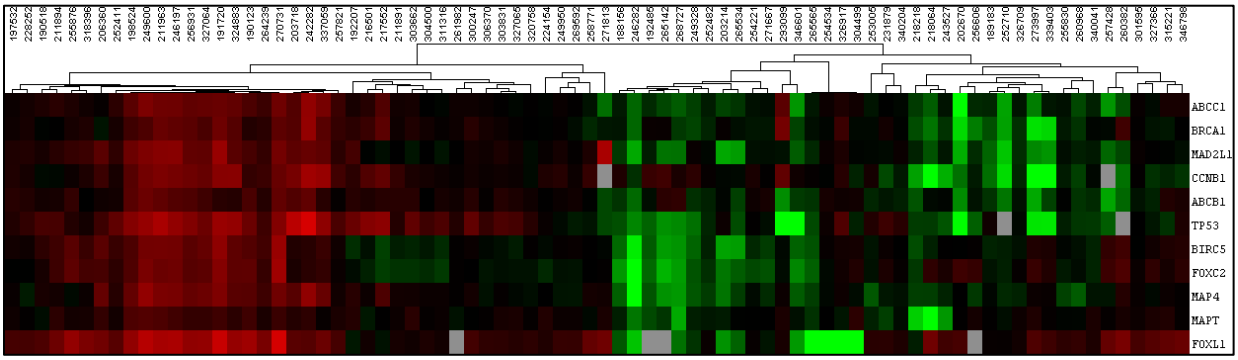


Figure 2.8 Heatmap showing hierarchical clustering of the LCL panel based on differentially expressed carboplatin pathway genes. a) All pathway genes; b) Pathway genes most significantly associated with cytotoxicity (p<0.1).
Columns represent cell lines; Rows represent carboplatin pathway gene analyzed using TLDA arrays.

a)



b)

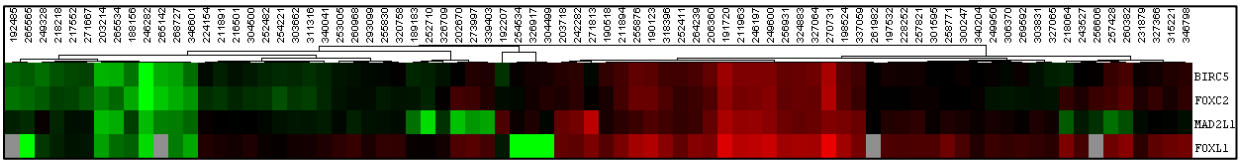


Figure 2.9 Heatmap showing hierarchical clustering of the LCL panel based on differentially expressed paclitaxel pathway genes. a) All pathway genes; b) Pathway genes most significantly associated with cytotoxicity (p<0.1).
Columns represent cell lines; Rows represent paclitaxel pathway gene analyzed using TLDA arrays.

2.4 Discussion and Conclusions

EOC accounts for 6% of all cancer deaths among women [3]. Despite the routine use of carboplatin-paclitaxel combination treatment, one of the biggest challenges faced by clinicians is wide inter-patient variation in clinical response, [7, 32] as well as toxicities including myelosuppression and gastrointestinal toxicities [7] and drug resistance [32]. Approximately 80% ovarian epithelial cancer patients relapse after first line based chemotherapy.

We hypothesized that modelling drug resistance using *in vitro* model systems will enable identification of genetic variants and gene expression signatures significantly associated with drug response. These drug response markers derived from *in vitro* models may then be validated using patient data to identify actionable genetic changes that influence drug resistance and treatment outcomes. In the current study, we used a panel of 81 LCLs derived from human ovarian cancer patients to identify key mutations and gene expression changes associated with carboplatin and paclitaxel response, both as single agents and when used as combination.

Our *in vitro* chemo-sensitivity assays showed wide variability in response to carboplatin and paclitaxel single agent and combination treatment, that mirrors the wide variability in clinical response among ovarian cancer patient samples. We also performed caspase activity assays to demonstrate that drug cytotoxicity reflected alterations in caspase-3 and caspase-7 activity, key effectors involved in cellular apoptosis and programmed cell death [33].

We then used Chou-Talalay's Combination index-Isobologram Theorem to understand the interactive effects of carboplatin and paclitaxel when used in combination treatment [31]. Evaluation of combination index, which is a direct measure of synergism, antagonism or additive interaction between the drugs revealed more than 25-fold variation across the cell line panel. Similar variation was also observed for DRI values, indicating that drug combinations are also variable based on the degree of refractoriness of a tumor. To understand the genetic determinants underlying this variability, we performed association analysis between the chemo-sensitivity parameters as

phenotype measures and mutations in genes involved in the PK/PD pathway of carboplatin and paclitaxel.

We identified significant association between carboplatin single-agent IC_{50} and genetic variations in drug transporter genes *ABCG2* and *ABCC2,* several mutations in the DNA repair gene *XRCC5* as well as mutations in *PMS2* and *MSH2*. Mutations in the following paclitaxel pathway genes were highly associated with paclitaxel IC_{50}: *TP53, EGFR,* and the transporter genes *ABCC1* and *SLC22A7*. On the other hand, several of the top genetic mutations associated with combination treatment belonged to the ATP-binding cassette transporter gene *ABCC1,* and the genes *MSH2* and *MSH6*. Notable among the associations we found between coding mutations and drug cytotoxicity were F150F in TP53 gene and the S667S in *ABCC1* that were associated with paclitaxel and combination treatment chemosensitivity, respectively. In addition, we also found association between caspase activity and the coding variants R704R *(ERCC6)*, R338R and P428P (MSH6) and carboplatin single-agent treatment. The genes *ABCC2* and *ABCG2* are involved in carboplatin efflux and multiple studies have shown association of these transporters with survival in ovarian cancer patients [9, 34]. Additionally, localization of *ABCC2* in the nuclear membrane was shown associated with cisplatin resistance and clinical outcome in ovarian carcinoma while *ABCC2* overexpression was associated with resistance to cisplatin in melanoma cells [35]. Similarly, *ABCG2* overexpression was found associated with poor survival for platinum-containing cancer chemotherapy [36]. In contrast, high *ABCC1* expression was associated with favorable survival measures[37]. Polymorphisms in *ABCC2* and *ABCG2* have been shown associated with response to platinating agents have shown correlated with treatment response and survival in ovarian cancer patients [38]. Polymorphisms in DNA repair genes may modulate not only DNA repair capacity, also clinical outcome of DNA damage-inducing anticancer drugs. For example, polymorphisms in *XRCC5,* a gene involved in non-homologous end joining repair process [39], have been shown associated with hematologic toxicity in cancer patients treated with platinum based compound [40].

PMS2, *MSH2* and *MSH6* are genes involved in DNA mismatch repair pathway that recognize and repair single stranded DNA damage due to Pt-DNA adducts [41]. We also found a non-synonymous coding variant K1087N in the gene *MAP4* significantly associated with caspase activity in paclitaxel single-agent treatment. Overexpression of *MAP4* have earlier been shown to increase microtubule polymerization and taxane binding with microtubule resulting in increased chemosensitivity [42, 43]. Overall, polymorphisms in these genes representing key nodes in the drug metabolism pathway that may have considerable impact on gene function and/or activity thereby affecting drug response.

We also performed differential gene expression of carboplatin and paclitaxel pathway genes to identify changes in gene expression levels associated with drug response. Significant among the differentially expressed carboplatin pathway genes were *ERCC2* (p=0.035 for IC_{50} and p=0.002 for AUC), *GSTM1* (p=0.010) and *NQO1* (p=0.032). Notably, earlier studies have shown deregulation of *GSTM1* enzyme activity influenced treatment response, 5-year survival and time to progression in various cancers including NSCLC and ovarian cancer patients [44]. Furthermore, differential expression of *GSTM1* also influences cisplatin sensitivity in cancer patients treated with platinum compound [45]. Upregulation of the nucleoside excision repair (NER) pathway genes ERCC1 and XPD expression was found significantly associated with higher resistance in platinum-based chemotherapy in advanced EOC [46, 47]. NER genes play significant role in the identification and repair of platinum-DNA adducts (1, 2-intrastrand DNA cross-links) [48].

Expression of the paclitaxel pathway genes that were significantly associated with cytotoxicity included *FOXC2* (p=0.003), *FOXL1* (p=0.011) and *BIRC5* (p=0.002). Alterations in the apoptotic gene *BIRC5* have been earlier shown associated with overall survival in patients receiving paclitaxel-cisplatin chemotherapy where OS was observed to decrease with increase in number of risk alleles ('bad genotypes') within DNA repair genes including BRCA1, XRCC1 and BIRC5 [49].

Although earlier studies have reported platinum and taxane sensitivity-related changes in DNA and gene expression using cell-based models, the modeling in these studies largely involved datasets that comprised of publicly available EBV-transformed LCL panels from healthy individuals including the Hapmap project [20-22] . Therefore, our study is the largest study so far conducted on LCLs modeled using cells derived directly from ovarian cancer patients.

Use of immortalized LCLs directly derived from patient subjects has several advantages over commercially available LCLs since it empowers us to develop *in vitro* drug response models that can be directly correlated with patient clinical responses. Here we successfully demonstrate that the use of candidate-gene based approaches in immortalized LCLs from patient samples can provide reliable genetic markers of drug responses/treatment outcomes that may eventually benefit clinical decision-making.

CHAPTER 3 RESPONSE TO PLATINUM, TAXANE, AND COMBINATION THERAPY IN OVARIAN CANCER: IN VITRO PHENOTYPES, INHERITED VARIATION, AND DISEASE RECURRENCE

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3.1. Introduction

Epithelial ovarian cancer (EOC) is the fifth leading cause of cancer death among women in the United States (6% of cancer deaths); in 2015, it is estimated that 14,180 women will die from the disease [1]. The standard treatment for patients with advanced disease is initial debulking surgery followed by carboplatin-paclitaxel combination chemotherapy [2]. Five-year overall survival remains around 45% [2], yet there is a wide inter-patient variation in response. Currently there are few reliable prognostic biomarkers for the classification of patients and treatment response.

Platinating agents, such as carboplatin, interfere with DNA via inter-strand, intra-strand, and DNA-protein crosslinks, thereby causing DNA damage and prevention of cell division and growth, resulting in cell-cycle arrest and apoptosis [3]. Although platinum-based drugs are widely used in cancer treatment, many tumors are completely resistant to these drugs, and no clinical response is attained. Major molecular mechanisms underlying this resistance might involve alteration in platinum inactivation or reduced intracellular accumulation by uptake/efflux transporters, increased repair of adducts, increased adduct tolerance or failure of apoptotic pathway. Taxane agents, such as paclitaxel, are commonly used chemotherapeutic drugs often in combination with platinating agents. Taxanes block cell division by binding to α-tubulin, stabilizing the microtubules, thus resulting in cell death [4, 5]. Although development of taxane resistance is common, the mechanism underlying this resistance is unclear. Molecules implicated in taxane metabolism and disposition include cytochrome P450s and drug transporters (e.g., ABCB1, ABCG2, ABCC1, ABCC2, and SLC01B3) [6, 7]. Inherited variation in many of the genes encoding these molecules has been assessed for association with clinical outcome with inconsistent results [8-10]; genome-wide searches to date have also failed to identify variants associated with outcome after correction for genome-wide testing ($p<5x10-8$).

Patient-derived cell line based model systems represent a novel way to identify genomic predictors of drug response. Although lymphoblastoid cell lines (LCLs) derived from participants in the

international HapMap project have been used to identify genomic predictors of cytotoxic effects of various chemotherapeutic agents [11-16], they are limited as they are not derived from the EOC population but from healthy individuals. Here, we generated LCLs derived from Mayo Clinic EOC patients, conducted *in vitro* cytotoxic studies, and associated *in vitro* drug response phenotypes with germline genotype. Utilizing patient-derived LCLs, as opposed to commercially available LCLs, allows us to screen and directly correlate *in vitro* phenotypes and clinical responses. These genome-wide association scans (GWAS) should contribute to the identification of predictive markers of treatment responses and ultimately improve clinicians' ability to tailor therapy decisions for EOC patients.

3.2. Materials and Methods

3.2.1. Patients, Lymphoblastoid Cell Lines and Cytotoxicity Assays

Prior to initiation of chemotherapy, ovarian cancer patients diagnosed at the Mayo Clinic between 2000 and 2003 provided blood for immediate germline DNA extraction and for the creation of Epstein Barr Virus (EBV)-transformed LCLs. Samples from 74 patients were successfully transformed and subjected to *in vitro* drug testing. All patients provided informed consent, including for passive and active follow-up, using protocols approved by the appropriate Institutional Review Board.

In vitro cellular chemo-sensitivity studies of LCLs were performed in two batches (N=33, N=41) using identical procedures and assays. Cells were maintained in RPMI1640 media supplemented with 2 mM L-glutamine, and 15% fetal bovine serum at 37°C under 5% CO_2. Following 24 hour incubation, LCLs were treated with increasing concentrations of carboplatin and/or paclitaxel (in duplicate). The concentrations of carboplatin were 0, 5, 10, 20, 40, 80 and 128 uM, while for paclitaxel were 0, 4.5, 7.5, 10, 20, 40 and 80 nM when used as single agents. In drug combination experiments, we used half of the doses for each drug in increasing doses (i.e. 2.5 uM carboplatin + 2.25 nM paclitaxel for "dose level 1" and so on). Cell viability 48 hours post-treatment was determined using standard MTT assay[13, 17, 18]. Caspase3/7 (Promega) apoptosis assays were performed at the same time in parallel plates [18, 19]. A Synergy 3 plate reader (BioTek Instruments) was used to read absorbance (cell viability using MTT) or fluorescence (for caspase3/7 activity) intensities.

Four parameter logistic dose response curves were fit to the *in vitro* drug response measurements (cell survival and caspase3/7 activity assays) for each LCL and treatment (paclitaxel, carboplatin, and combination):

$$Yi = \alpha + (\beta - \alpha)) / (1 + [Di / \theta]^{\phi})$$

where Yi is the measurement at dose i (Di), α is the estimated bottom of the curve (i.e., measurement as Di $\rightarrow \infty$), β is the estimated top of the curve (i.e., measurement as Di $\rightarrow 0$), ϕ is the slope of the curve, and θ is the inflection point of the curve (i.e., concentration giving a response half way between the top and bottom of the curve). The estimated inflection point of the curve was used as the drug response phenotype. That is, using these dose-response curves, we estimated the relative IC_{50} (effective dose that kills 50% of the cells) for MTT cell viability and the relative EC_{50} (concentration required to induce caspase 3/7 activity by 50%) for caspase3/7 activity [20]. For simplicity of presentation, we will refer to these quantitative values as the IC_{50} for the MTT cytotoxicity assays and EC_{50} for the caspase 3/7 assays. We then applied rank-based inverse Gaussian transformation (i.e., Van de Waerden rank transformation).

Summaries of the IC_{50} and EC_{50} values for both experimental batches are presented in **Table 3.1**. We observed a high degree of correlation between many of the drug response phenotypes, as expected (**Figure 3.1**).

Table 3.1 Mean (SD) *in vitro* phenotype measures collected on LCLs from 74 EOC patients and comparison across experimental batch.

Phenotype	Treatment	Batch 1 (N=33)	Batch 2 (N=41)	P*
MTT IC_{50}	Paclitaxel	14.22 (8.4)	27.66 (55.1)	0.131
	Carboplatin	55.16 (73.9)	105.52 (138.0)	0.049
	Combination	31.10 (37.6)	47.16 (84.8)	0.281
Caspase 3/7 EC_{50}	Paclitaxel	11.34 (6.7)	9.39 (6.2)	0.865
	Carboplatin	26.00 (13.9)	26.58 (15.3)	0.203
	Combination	10.36 (4.39)	10.28 (5.9)	0.953

*P is result from testing difference in two groups based on a two-sample t-test assuming unequal variances (Welch Test)

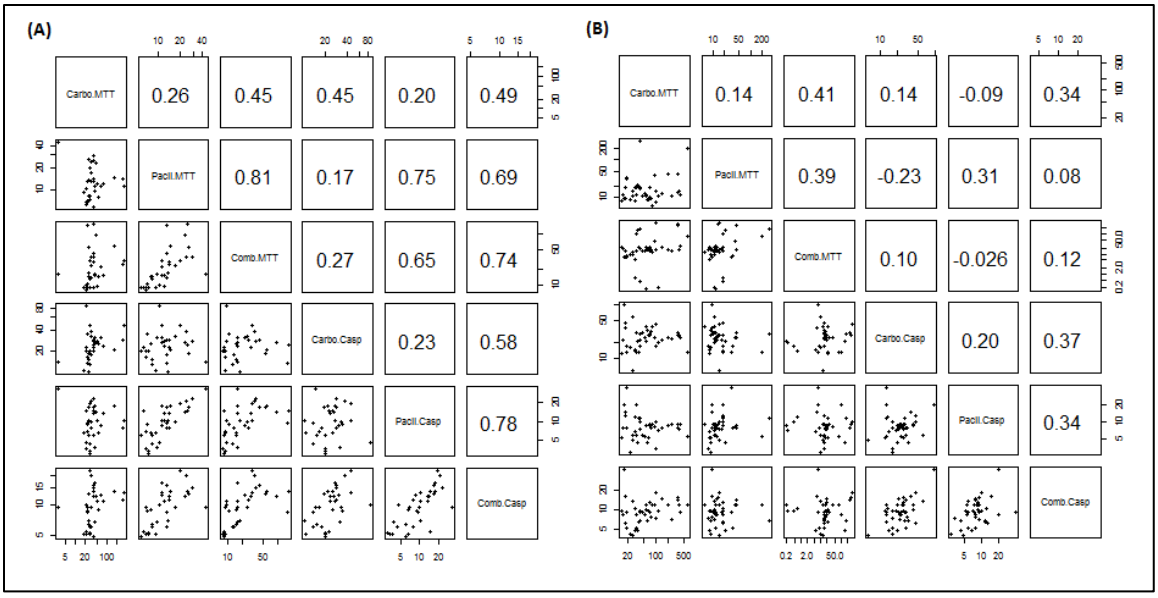

Figure 3.1 Scatterplot matrix of drug response phenotypes by experimental batch.
Spearman correlations are presented on the upper panel of each plot (log scale). (A) Batch 1 (N = 33), the highest correlation being between paclitaxel IC_{50} and combination IC_{50} (r = 0.81) and caspase EC_{50} measurements for paclitaxel and combination treatments (r = 0.78). (B) Batch 2 (N = 41), the highest correlation was between paclitaxel and combination IC_{50} measurements (r = 0.39).

3.2.2. Genotyping and Statistical Methods

Germline DNA was genotyped on the Illumina Infinium 610K array, as previously described [19, 21, 22]. All samples had genotype call rate >95% and were predicted by STRUCTURE [23] analysis to have greater than 80% European ancestry. SNPs were excluded with call rate < 95%, Hardy-Weinberg Equilibrium p < 10-4, or no variation in this set. Using the 1000 Genomes Project [24] as reference, imputation was completed with *mach* and *minimac* in a two-step process [25], resulting in data on more than 30 million SNPs. Assessment of imputation quality was completed and high quality imputed markers ($r2 \geq 0.30$ and $MAF \geq 0.01$) were retained (6,243,550 SNPs).

The association of each SNP with *in vitro* drug response phenotypes IC_{50} or EC_{50} was evaluated with linear models [26] using the expected genotype or "dosage" (i.e., additive or dose-response/trend model). Thus, a negative effect estimate indicates that the carriers of the minor/rare allele have lower IC_{50} (EC_{50}) values (i.e., were more "sensitive" to treatment). The two *in vitro* experimental batches were analyzed separately followed by meta-analysis was conducted using metal [27], with

weights applied for the number of samples in each group. We completed the GWAS analyses for each individual *in vitro* drug response phenotype, as opposed to a combined analysis with all phenotypes model together, due to the difference in mechanism of action between the drugs (i.e., not in the same drug class) [28]. For annotation of results across gene regions, SNPs were mapped to genes within 2KB using Biofilter (assembly CRCh37.p10, genome build 104.0) [29]. Pathway analysis used Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com).

3.3. Results

We examined the relationships of *in vitro* phenotypes with time to recurrence of EOC (40 of 74 patients had recurred or died). Of the 74 patients with LCLs included in this study, 51 had available information on the first two treatments used: 43 patients were treated with paclitaxel / carboplatin, 2 treated with paclitaxel / cisplatin, 3 treated with carboplatin / topotecan, and 3 treated with carboplatin / other less common agent. paclitaxel *in vitro* drug response phenotypes were moderately associated with time to EOC recurrence (HR = 1.90 per unit increase in MTT IC_{50}, p = 0.008; HR = 1.84 per unit increase in caspase 3/7 EC_{50}, p = 0.058) (**Figure 3.2**) (**Table 3.2**).

This suggests that patients whose LCLs demonstrated greater sensitivity to the chemotherapeutics tested had improved outcome; as **Figure 3.2** illustrates, LCLs that were sensitive to paclitaxel (as reflected by having low IC_{50} and low EC_{50} values) were from patients with longer time to progression as compared to patients with LCLs with high values. Although based on a small sample size, this provides, for the first time, a link between *in vitro* chemo-sensitivity testing and clinical outcome in EOC.

Results of genome-wide association analyses for each drug response phenotype are presented in **Figure 3.3**. Regions with p < 10-6 are highlighted and are further displayed in **Figure 3.3**. **Table 3.3** presents the SNPs associated with the drug response phenotype with p < 10-6. Overall, we found a greater proportion of significant results (e.g., at p < 10-6) for the combination therapy as compared to the single agent therapies. In particular, we found strong SNP associations with combination therapy in the following gene regions: SLC9A9 (MAF = 0.41, p = 6x10-7), TIAL1 (MAF = 0.23, p = 7.3x10-7), ZNF731P (MAF = 0.39, p = 6.6x10-7), and PCDH20 (MAF = 0.42, p = 8.2x10-7). None of these regions were found to be moderately associated with single agent carboplatin or paclitaxel IC_{50} in other pharmacogenomic studies involving commercially available LCLs, such as those of the International HapMap Consortium (personal communications for carboplatin study and paclitaxel study published by Niu et al [30].

Table 3.2 Hazard ratios (p-value) associating *in vitro* drug phenotypes with time to disease recurrence.

	Caspase 3/7 EC_{50}	MTT IC_{50}
Paclitaxel	1.84 (0.058)	1.90 (0.008)
Carboplatin	1.00 (0.991)	1.00 (1.000)
Combination	1.42 (0.376)	1.01 (0.930)

* P-value determined from a likelihood ratio test; Hazard Ratio > 1 indicates worse outcome for subjects with high IC_{50} ("resistant"); 40 events; bold, p<0.10

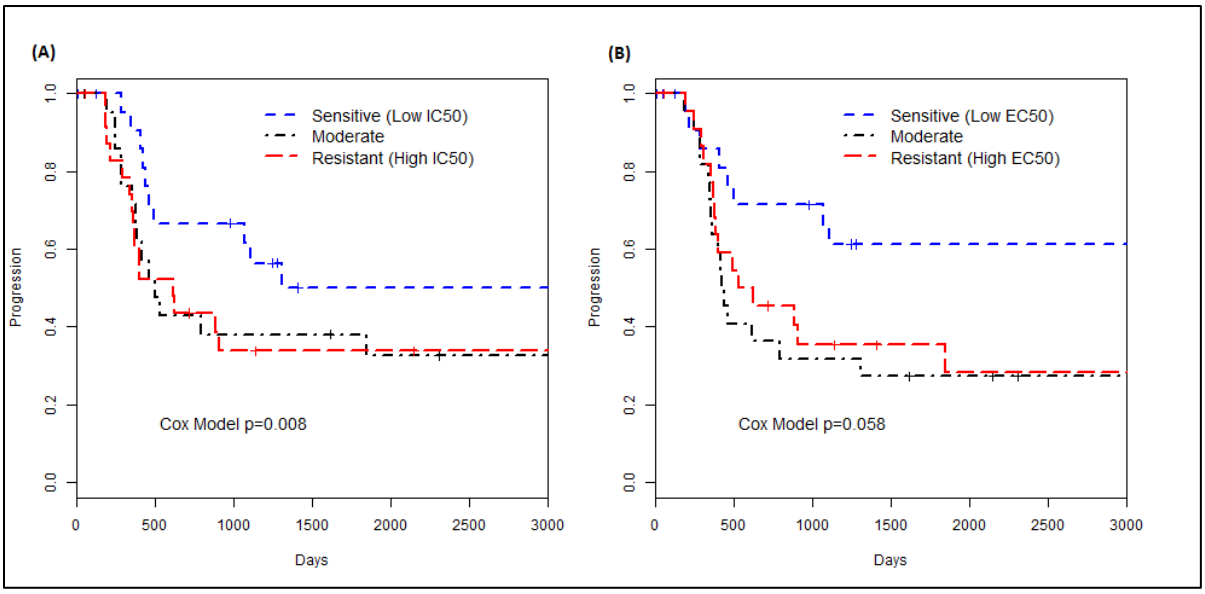

Figure 3.2 Kaplan-Meier curves for time to progression and paclitaxel *in vitro* phenotypes. The groupings were defined by 3 quartiles representing low, medium or high values for the phenotype based (A) MTT assay IC_{50} or (B) Caspase 3/7 EC_{50} assay. The p-value presented is from a Cox proportional hazards model with the *in vitro* phenotype modeled as a continuous measurement on the log-scale.

Table 3.3 SNPs with $p < 10^{-}6$ association with a drug response phenotype.

Drug	Phenotype	Nearest Gene	SNP	Chr	Position	MAF	Meta-Analysis P	Group 1 (N=33)		Group 2 (N=41)	
								Estimate	P	Estimate	P
Paclitaxel	MTT IC_{50}	*BOD1L1*	rs185229225	4	13609129	0.02	2.2E-07	-2.53	8.0E-03	-11.64	4.7E-06
	Caspase 3/7 EC_{50}	*MGC32805 /SNCAIP**	rs3842595	5	121778606	0.14	2.6E-07	-1.37	1.7E-03	-1.34	4.1E-05
Carboplatin	MTT IC_{50}	*FRAS1*	rs150303591	4	79009309	0.29	5.9E-07	0.86	2.5E-03	1.02	6.3E-05
Combination	MTT IC_{50}	*SLC9A9**	rs201023017	3	143103669	0.41	6.0E-07	0.84	5.2E-04	0.70	3.3E-04
		TIAL1	rs66696671	10	121366953	0.23	7.3E-07	-0.89	2.9E-04	-0.76	6.6E-04
	Caspase 3/7 EC_{50}	*ZNF731P*	rs12025262	1	247356732	0.39	6.6E-07	-0.84	2.5E-04	-0.71	6.9E-04
		PCDH20	rs10674174	13	61892075	0.42	8.2E-07	-0.73	5.3E-03	-0.86	3.7E-05

*Nearest gene within 2000 gene pairs.

For regions with multiple SNPs with $p < 10^{-6}$, the most significant SNP is presented. A negative estimate indicates that carriers of the minor allele had, on average, lower IC_{50} or EC_{50} ("sensitive") while a positive estimate indicates that carriers of the minor allele had, on average, higher IC_{50} or EC_{50} ("resistant").

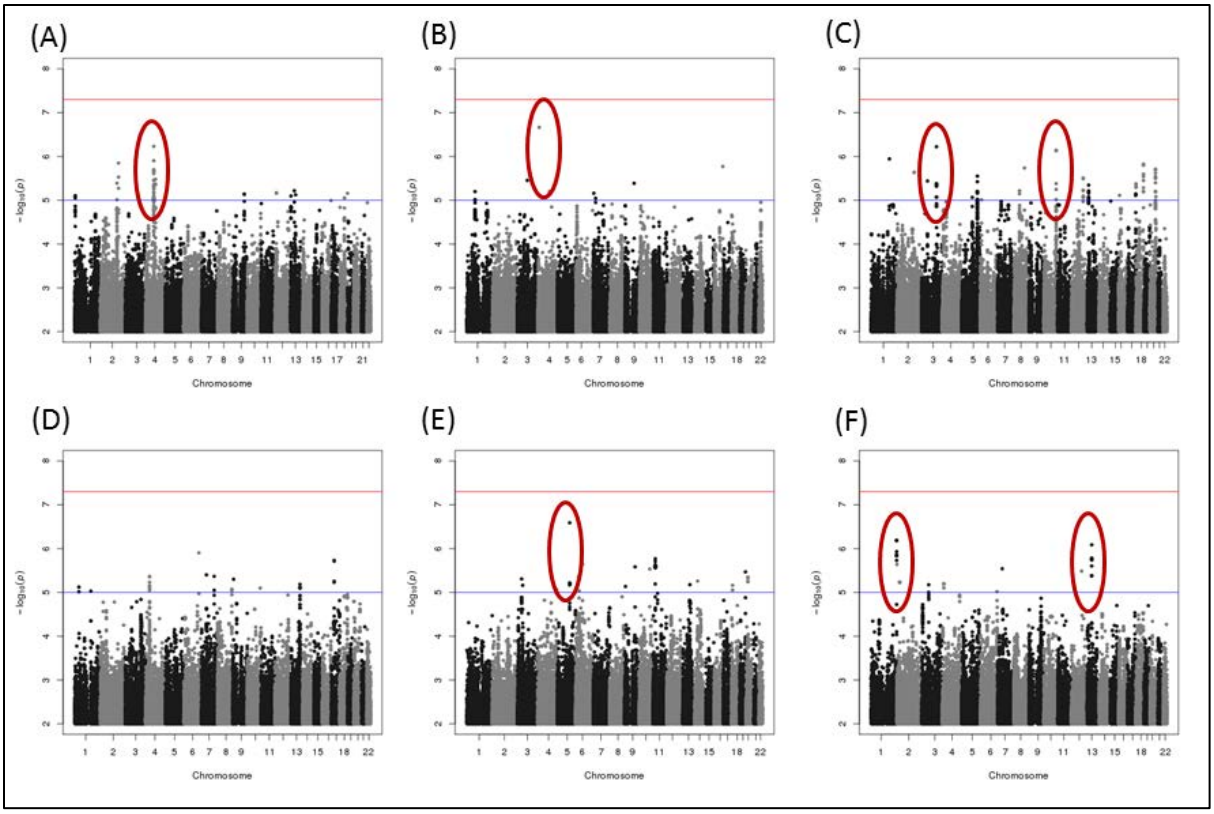

Figure 3.3 Manhattan plots of the single SNP meta-analysis genome-wide association results based the six *in vitro* drug response measures among ovarian cancer patient LCLs.

(A) carboplatin MTT; (B) paclitaxel MTT; (C) Combination of carboplatin and paclitaxel MTT; (D) carboplatin Caspase3/7; (E) paclitaxel Caspase3/7; and (F) Combination of carboplatin and paclitaxel Caspase3/7. Blue line indicates p-value = 0.00001; Red line indicates p-value=5x10-8. Highlighted regions (circled) are displayed in **Figure** 3.

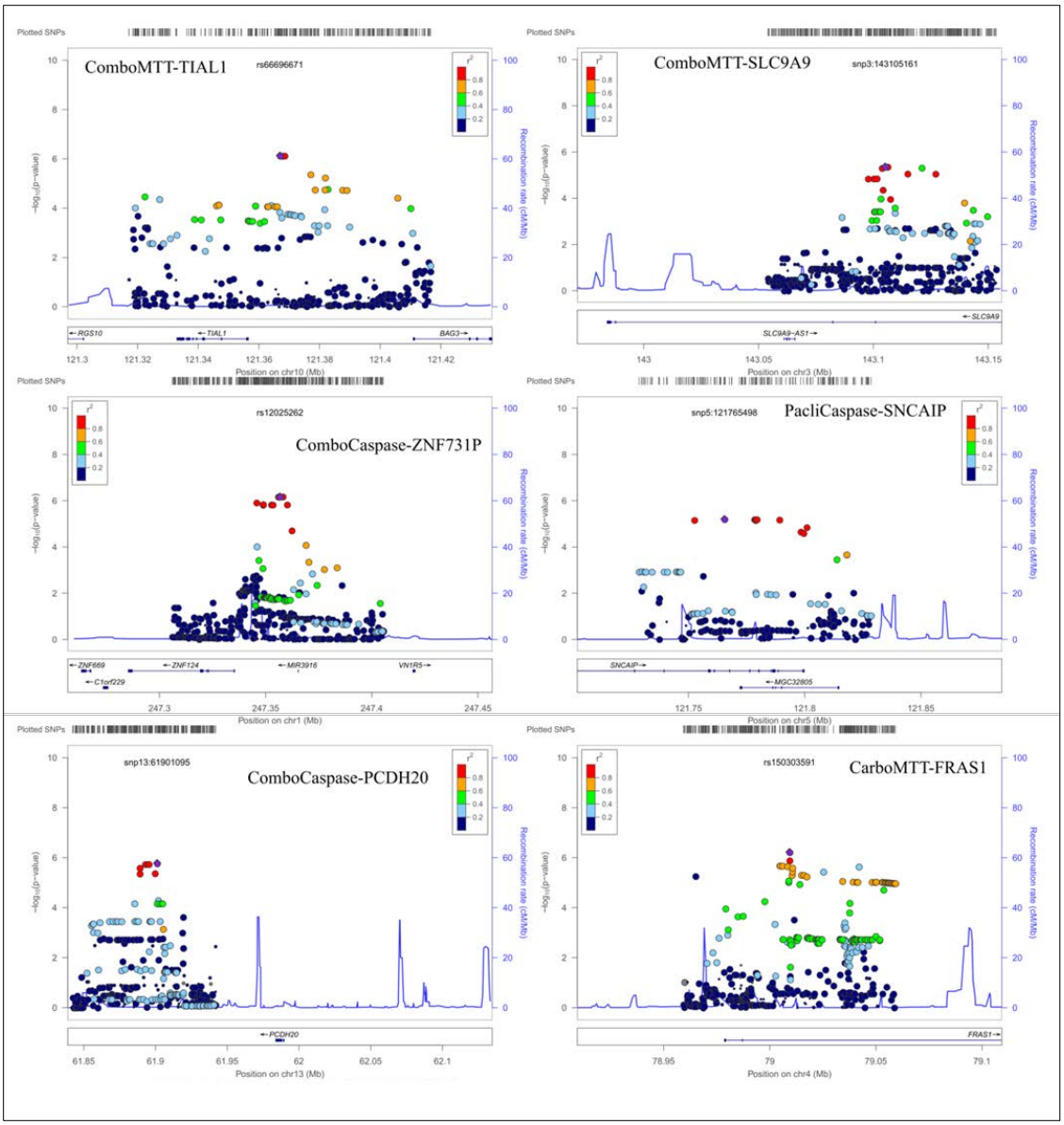

Figure 3.4 Locus Zoom plots for regions with $p < 10^{-6}$.

Note: BOD1L1 region not presented as only one SNP in region with p < 0.001.

We evaluated potential overlap of loci associated with both phenotypes for a given drug. Only one SNP was found to be associated with a p < 10-4 with same direction of the effect for MTT IC_{50} and caspase3/7 EC_{50} values for paclitaxel, carboplatin, or combination treatment. An intronic SNP rs35067965 in COLEC12 (chromosome 18, bp 455396) was associated with response to paclitaxel (MTT IC_{50} p = 2.2 x 10-5, caspase 3/7 EC_{50} p = 3.8 x 10-5) (**Table 3.4**).

We also examined overlap of associations at the level of genes, considering SNPs within 20kb. This showed consistency of IC_{50} and EC_{50} results for paclitaxel response and COLEC12 and revealed similar IC_{50} and EC_{50} associations for carboplatin response in the gene regions of CTIF and CDH4. As presented in **Table 3.5**, additional regions showed joint associations with response to multiple drugs, including variants in protein coding regions of BRE, EML6, CTNNA2, LRP1B, EYS, NKAIN2, ANTXRL, COL13A1, and MTCL1 (SNPs in gene regions with $p < 0.0001$).

Because of the suggested association between *in vitro* paclitaxel MTT IC_{50} response and time to EOC recurrence (**Table 3.2**), we also examined recurrence association with SNPs rs185229225 (intronic BOD1L1) rs35067965 (intronic COLEC12) and rs1525599 (intronic LRP1B) which were associated with paclitaxel MTT IC_{50} (**Table 3.3., Table 3.4. and Table 3.5**., respectively). However, none of these SNPs were associated with time to recurrence with a nominal p-value < 0.05 (data not shown).

Table 3.4 Gene regions with SNPs associated with both phenotypes for a given drug.

Drug	Gene	Phenotype	SNP*	Chr	Position	P	Direction†
Paclitaxel	*COLEC12*	MTT IC_{50}	rs35067965	18	455396	2.2E-05	--
		Caspase 3/7 EC_{50}	rs35067965	18	455396	3.8E-05	--
Carboplatin	*CTIF*	MTT IC_{50}	rs8091660	18	46087936	8.9E-06	--
		Caspase 3/7 EC_{50}	rs113867814	18	46259604	1.2E-05	--
	CDH4	MTT IC_{50}	rs2748151	20	60133486	4.7E-05	++
		Caspase 3/7 EC_{50}	rs113594423	20	60379048	2.4E-05	++

*Presenting most significant SNP in the region for the giving drug/phenotype. SNP within ± 20 KB of the listed gene. †A positive estimate indicates that carriers of the minor/variant allele had, on average, higher IC_{50} or EC_{50} ("resistant").

Table 3.5 Gene regions with SNPs associated with multiple drugs for any phenotype ($p < 0.0001$).

Gene	Drug	Phenotype	SNP*	Chr	Position	P	Direction†
BRE	Carboplatin	Caspase 3/7 EC_{50}	rs5830067	2	28537890	1.70E-05	++
	Combination	Caspase 3/7 EC_{50}	rs7572644	2	28320033	5.80E-06	--
EML6	Paclitaxel	Caspase 3/7 EC_{50}	rs75314082	2	55087315	7.90E-05	--
	Combination	MTT IC_{50}	rs17046344	2	55023600	4.90E-05	++
LINC01122	Paclitaxel	Caspase 3/7 EC_{50}	rs72817940	2	58998563	6.40E-05	++
	Carboplatin	Caspase 3/7 EC_{50}	rs4233974	2	59295043	2.60E-05	--
CTNNA2	Carboplatin	MTT IC_{50}	rs17261321	2	80197843	3.60E-05	++
	Combination	MTT IC_{50}	rs6719499	2	80193386	6.00E-05	--
LRP1B	Paclitaxel	MTT IC_{50}	rs1525599	2	141778702	8.60E-05	++
	Combination	Caspase 3/7 EC_{50}	rs13020675	2	142212928	6.20E-05	--
EYS	Paclitaxel	Caspase 3/7 EC_{50}	rs201083182	6	65736914	2.30E-06	--
	Combination	Caspase 3/7 EC_{50}	rs2064701	6	65676556	3.60E-05	++
NKAIN2	Paclitaxel	Caspase 3/7 EC_{50}	rs550987	6	124905510	4.10E-05	--
	Combination	Caspase 3/7 EC_{50}	rs670616	6	124885773	7.80E-05	++
C7orf65	Carboplatin	Caspase 3/7 EC_{50}	rs10230114	7	47705506	2.40E-05	++
	Combination	Caspase 3/7 EC_{50}	rs11771997	7	47712495	2.40E-05	++
ANTXRL	Paclitaxel	Caspase 3/7 EC_{50}	rs12572446	10	47665906	4.30E-05	++
	Combination	Caspase 3/7 EC_{50}	rs10906942	10	47670851	4.90E-05	++
COL13A1	Carboplatin	Caspase 3/7 EC_{50}	rs10999018	10	71654602	2.40E-05	++
	Combination	Caspase 3/7 EC_{50}	rs77535242	10	71652985	3.50E-05	++
TMEM132D	Paclitaxel	Caspase 3/7 EC_{50}	rs77438645	12	130304313	7.60E-05	--
	Carboplatin	Caspase 3/7 EC_{50}	rs1451904	12	130166947	6.50E-05	++
MTCL1	Carboplatin	Caspase 3/7 EC_{50}	rs690089	18	8845223	7.80E-05	--
	Combination	Caspase 3/7 EC_{50}	rs35765215	18	8839469	6.00E-05	--

*Presenting most significant SNP in the region for the giving drug/phenotype. SNP within ±20 KB of the listed gene. †A negative estimate indicates that carriers of the minor/variant allele had,on average,lowerIC_{50} or EC_{50}("sensitive") whilea positive estimate indicates that carriers of the minor/variant allele had,on average,higher IC_{50} or EC_{50}("resistant").

3.4. Discussion and Conclusions

In this proof of concept study, we explored use of LCLs derived from EOC patients followed for clinical response as a model for discovery of pharmacogenomics markers. LCLs were treated with varying concentration of the chemotherapeutics agents (carboplatin and paclitaxel and, uniquely, their combination) that were used for the treatment and cellular chemo-sensitivity was determined by measuring cell viability and activation of caspase activity (as a marker of apoptosis) post drug treatment. Genome-wide association studies were performed to identify inherited markers associated with these measures of *in vitro* chemo-sensitivity (i.e., MTT IC_{50} and caspase 3/7 EC_{50} values) and the relationships between *in vitro* measures and clinical outcome was explored.

Although the sample size was small limiting the power of the study, some of the implicated biologically interesting genes are worthy of discussion. Pathway analysis of genes with SNPs showing association with one of the drug response phenotypes (at $p < 10\text{-}6$), both phenotypes for a given drug (at $p < 10\text{-}4$), or multiple drugs for any phenotype (at $p < 10\text{-}4$) found enrichment in genes related to malignant solid tumor and epithelial cancers (**Figure 3.5A**). Among the top canonical pathways represented by these genes were "Epithelial Adherens Junction Signaling", "Sertoli Cell Junction Signaling", and "Endometrial Cancer Signaling" (**Figure 3.5B**). In addition, genes such as CTNNA2 and CDH4, both tumor suppressor genes with role in cell adhesion were found to be associated with chemo-sensitivity in carboplatin alone or combination treatments. SNPs in CTNNA2- catenin (cadherin-associated protein) alpha2, a structural constituent of cytoskeleton and cadherin binding was associated with *in vitro* cytotoxicity to carboplatin alone as well as in combination with paclitaxel. CTNNA2 has been shown to be frequently mutated in laryngeal carcinomas with mutations predictive of poor prognosis [31]. Additionally, SNPs within CTNNA2 have recently been implicated in breast cancer [32] and its role in tumor progression and metastasis has been suggested for multiple cancers [33]. Variants in CTNNA2 have also been implicated in schizophrenia [34] and alcohol addiction [35]. CTNNA2 SNPs associated with carboplatin and

paclitaxel MTT IC_{50} were both intronic and present functional relevance of these is not known. CDH4, codes for cadherin, and has been implicated in nasopharyngeal carcinoma [36] and aberrant methylation of CDH4 promoter has been colorectal and gastric cancer [37]. Our results identified two intronic SNPs (rs2748151 and rs113594423) that were associated with carboplatin resistant as measured by cell death (IC_{50}) and apoptosis (caspase 3/7 EC_{50}). Variants in PCDH20, another member of cadherin family, were also found to be associated *in vitro* drug response. PCHD20 codes for protocadherin20 and functions as a tumor suppressor by interacting with Wnt/b-catenin signaling[38, 39].

Another gene with role in cell adhesion identified in our study was FRAS1, which encodes for an extracellular matrix protein and is involved in the regulation of epidermal-basement membrane adhesion and organogenesis during development. Inherited mutations in FRAS1, and FREM2, have been associated with development of Fraser syndrome. FRAS1 has also been implicated in ERK signaling and influence migration and invasion of lung cancer cell line by influencing FAK signaling [40], suggesting its role in tumorigenesis and metastasis of lung cancer. Although the genes described above are involved cell adhesion/cell migration, the functional significance of the intronic SNPs identified in this study is not known and would require further investigation.

Two intronic variants within BRE were found to be associated with caspase 3/7 levels for carboplatin and combination therapy (indel rs5830067 and rs7572664). BRE encodes for Brain and reproductive Organ-Expressed (TNFRSF1A modulator) and is a component of BRCA1-A DNA damage repair complex that recognizes Lys 62linked ubiquitinated H2A and H2Ax at DNA lesions, resulting in recruitment of BRCA1-BARD1 to double strand DNA breaks [41]. BRE expression has been shown to be predictive of disease free survival in non-familial breast cancer patients [42] and recent studies show its involvement in both intrinsic and extrinsic apoptotic pathways by influencing XIAP [43]. Variation within EML6, which is involved assembly dynamics of microtubules, was found to be associated with platinum-sensitivity which was of interest since

paclitaxel's mechanism of action involved disruption of microtubules; however no evidence exists in the literature on functional relevance of these particular SNPs within EML6. No other genes involved in microtubule protein were identified with respect to paclitaxel chemo-sensitivity.

Lastly, two intronic variants within LRP1B (low density lipoprotein related protein 1B) were associated with paclitaxel and combination therapy drug response phenotypes. LRP1B is a tumor suppressor with decreased expression in several primary cancers and is among ten most significantly deleted genes across 3312 cancer samples [44-47]. In renal cell cancer, down-regulation of LRP1B has been shown to regulate cell motility and actin cytoskeleton reorganization [48]. Germline SNPs/ haplotype in LRP1B have been associated with aging without cognitive decline [49]; however, associations of germline SNPs with incidence/progression of cancer and pharmacogenomics have yet to be reported.

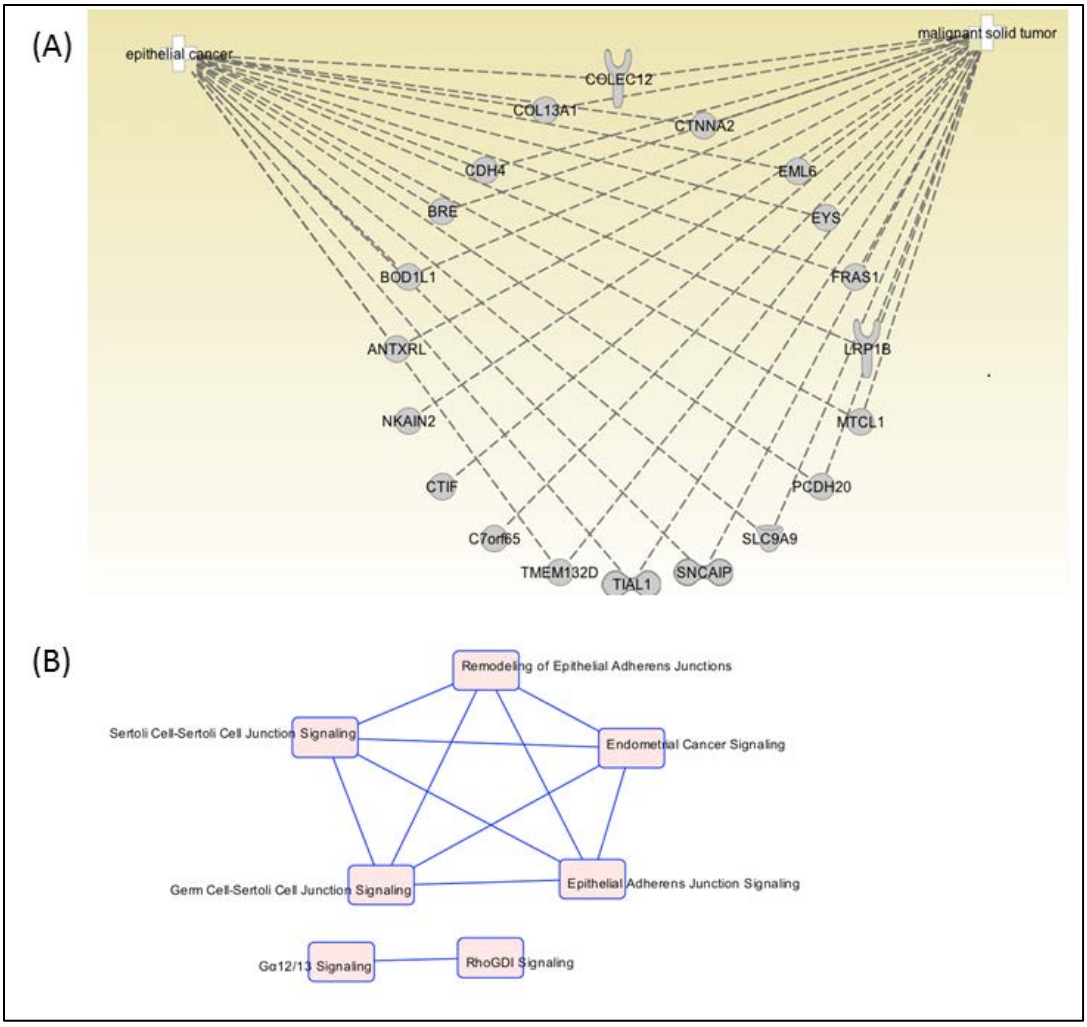

Figure 3.5 Pathway analysis of genes with SNPs that demonstrated significant association with LCL chemosensitivity with carboplatin or paclitaxel alone or in combination.

A) Significant number of genes mapped to malignant solid tumor or epithelial cancer; B) Top canonical pathways and interaction network among the pathways represented by significantly associated genes.

In summary, using a patient-derived cell-based model system to generate several *in vitro* drug response phenotypes on a clinically followed set of EOC cases we have identified genetic loci associated with response to platinum-taxane therapies. Overall our results identified germ-line SNPs in multiple cell adhesion molecules and several tumor suppressor genes (PCDH20, LRP1B, CDH4, and CTNNA2). However, none of the most associated SNPs were reported by Huang et al [15] or associated with mRNA gene expression in lymphoblastoid cell lines (http://www.ncbi.nlm.nih.gov/projects/gap/eqtl/index.cgi). Further studies are needed to determine if these SNPs are truly associated with drug response or if they represent false-positive

findings. Similar to other studies comparing *in vitro* chemo-sensitivity with clinical outcomes [15, 50], our findings suggest that *in vitro* response to paclitaxel correlates with time to disease recurrence indicating that this model may have utility in several types of future studies. On possible explanation for the observation that paclitaxel correlates with recurrence and not carboplatin may be the fact that the majority of EOC patients eventually develop platinum resistant tumors and the main factor related to future response maybe attributed to response to taxane therapy. Further research is needed to understand the mechanism by which genomic loci impact clinical response in ovarian cancer patients to the most common regimen used in the treatment of ovarian cancer following surgery.

CHAPTER 4 GENETIC VARIATION IN PLATINATING AGENT AND TAXANE PATHWAY GENES AS PREDICTORS OF OUTCOME AND TOXICITY IN ADVANCED NON-SMALL-CELL LUNG CANCER

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4.1 Introduction

Lung carcinoma is the most common malignancy worldwide, and the leading cause of cancer deaths. According to the most recent estimates, the global incidence of lung cancer is more than 1.6 million cases/year, resulting in more than 1.3 million deaths/year (>18% of all cancer deaths) [1]. In the USA, the incidence of lung cancer was estimated to be 228,000 cases in 2013, leading to nearly 160,000 deaths (27.5% of all cancer deaths) [2]. Histologically, the majority (approximately 80%) of lung cancers are non-small-cell carcinomas (NSCLCs). The 5-year relative survival of patients with advanced NSCLC remains dismal at approximately 4% [2]. Indeed, the median survival of unselected patients diagnosed with metastatic NSCLC is only 4 months [3]. Platinating agent-based combination chemotherapy is the standard treatment for patients with advanced NSCLC who have adequate organ function and performance status, and whose tumor does not have a driver mutation amenable to treatment with a specific inhibitor. However, combination chemotherapy achieves objective response in only approximately 26% of patients, improves survival modestly, and is associated with diverse side effects [4]. Interpatient variation in response and toxicity, which cannot be predicted for individual patients, precludes the selection and tailoring of chemotherapy that might improve outcomes and minimize adverse events. Although clinical factors including age, performance status and stage influence the likelihood of benefit from and tolerability of chemotherapy, the genetic profile of individual patients may contribute significantly to the marked variation in response and toxicity.

Adverse effects associated with platinating agent-based combination chemotherapy include gastrointestinal toxicity, myelosuppression, nephrotoxicity, peripheral neuropathy and ototoxicity [5].Platinating drugs such as cisplatin and carboplatin act by forming platinum–DNA adducts, which lead to cell cycle arrest and apoptosis. Several genes are involved in the carboplatin drug pathway (platinating agents pathway PharmGKB: (**Figure 4.1**) Intracellular levels of carboplatin are regulated by drug transporters SLC31A1 (CTR1), ABCC2 (MRP2), ATP7A and ATP7B [6], as well

as drug-metabolizing enzymes including MPO, SOD1, GSTM1, NQO1, GSTP1 and MT, which are implicated in the development of cellular resistance to these drugs [7-10]. Genes of pharmacodynamic significance include HMGB1, which is involved in recognition and cellular response to platinum–DNA, adducts and DNA repair genes including mismatch repair genes MSH6and MLH1, and nucleotide excision repair genes XRCC1, ERCC1, ERCC2and XPA [11, 12]. Genetic variation in these genes of importance to pharmacokinetic and pharmacodynamics pathways of platinating agents may thus contribute to interpatient variation in response and tolerability [13].

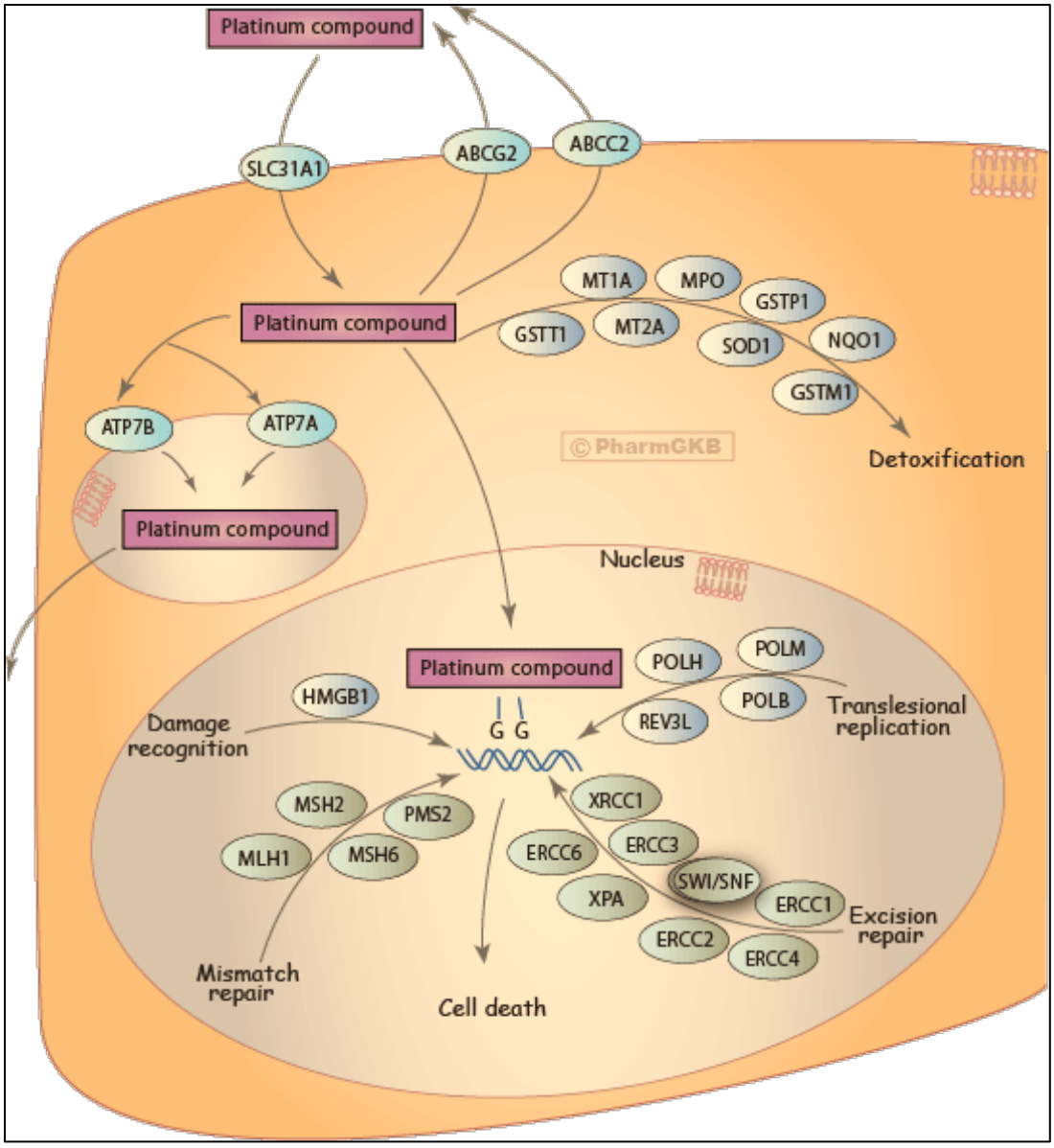

Figure 4.1 Platinum pharmacokinetics/pharmacodynamics pathway
Representation of the candidate genes involved in the metabolism of platinum containing drugs [14].

Taxanes are routinely given in combination with platinating agents, genes involved in efflux (ABCC1, ABCC2, ABCG2 and ABCB1) and metabolism of taxane (CYP3A4 and CYP2C8) are critical for its therapeutic efficacy (taxane pathway: PharmGKB) (**Figure 4.2).** Although the role of genetic variation in taxane response is unclear at this time, some studies have found no significant association between SNPs and treatment outcome [15], whereas others have found

significant associations between ABCB1SNPs and response to paclitaxel [16], and gastro intestinal toxicity in patients treated with taxane and platinum combination therapy [17]. In the present study, we evaluated SNPs in genes of relevance to the pharmacokinetic/pharmacodynamics pathways of platinating agents and taxanes in patients diagnosed with advanced NSCLC and treated primarily with carboplatin-based doublet chemotherapy, and determined the association of individual SNPs with outcomes and toxicity. We identified specific SNPs that were predictive of progression-free survival (PFS) and multiple adverse effects, after adjusting for known clinical prognostic factors in multivariate models. Results of our proof-of-concept study provides evidence that in real world clinical settings the association of genetics with clinical outcome is evaluable and although validation in larger cohorts is required, genetic information can be utilized to develop to more effective treatment strategies.

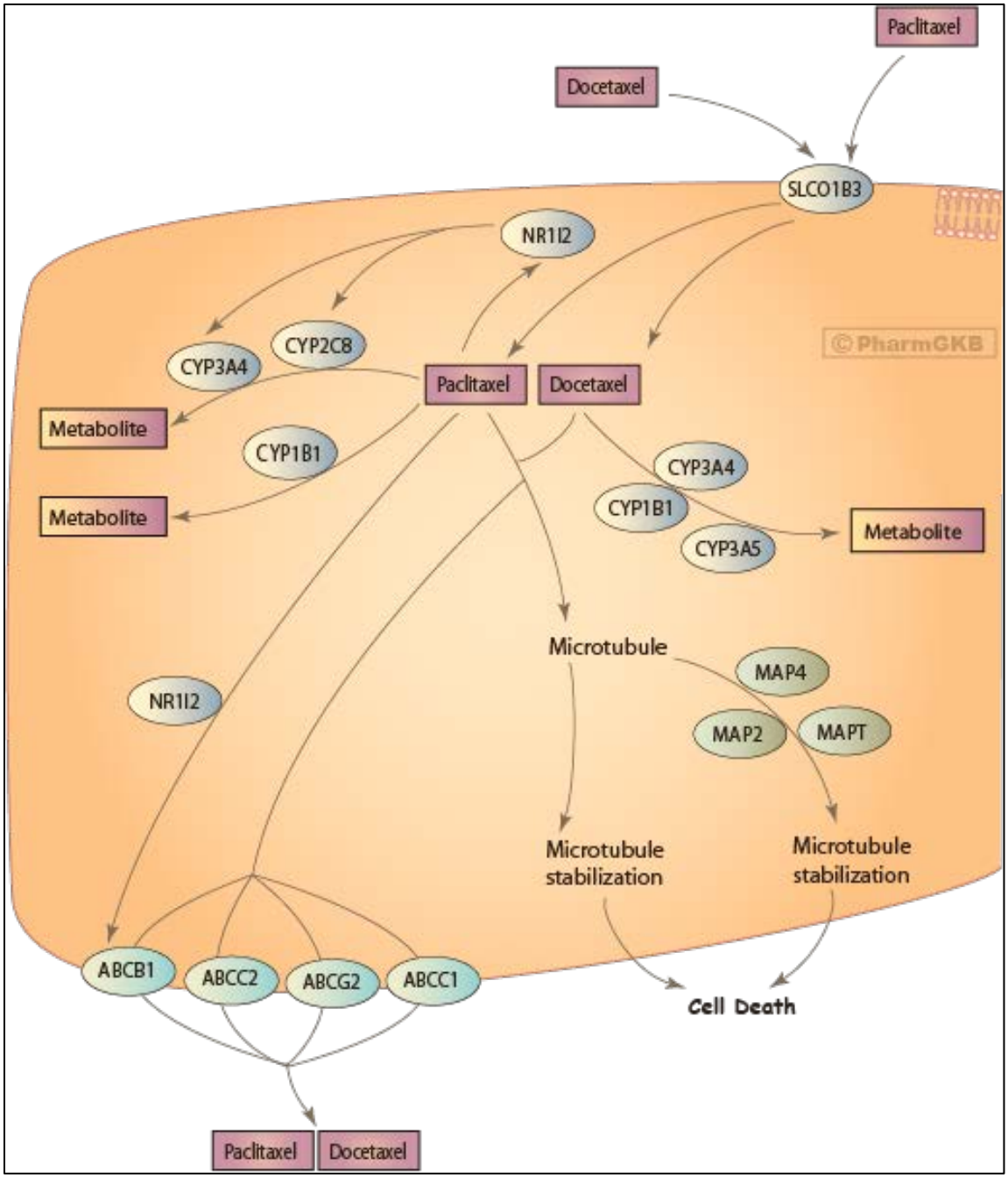

Figure 4.2 Taxane pharmacokinetics pathway

Representation of the genes involved in the metabolism and transport of paclitaxel and docetaxel, and the downstream effects of the drugs [18].

4.2 Materials and methods

4.2.1 Patients cohort

A total of 635 patients diagnosed with stage IIIB or IV NSCLC between December 1998 and December 2008 were identified by the institutional tumor registry (**Figure 4.3**). Of these, 90 patients met the following eligibility criteria: histological or cytological confirmation of NSCLC, presence of measurable disease, performance status Eastern Cooperative Oncology Group (ECOG) 0–2, no neoadjuvant or concurrent radiation therapy or surgery, no second malignancies, availability of adequate diagnostic tumor tissue, treatment with first-line platinating-agent based doublet chemotherapy and complete follow-up at the Minneapolis VA Health Care System (MN, USA). The study was approved by the institutional human subjects committee.

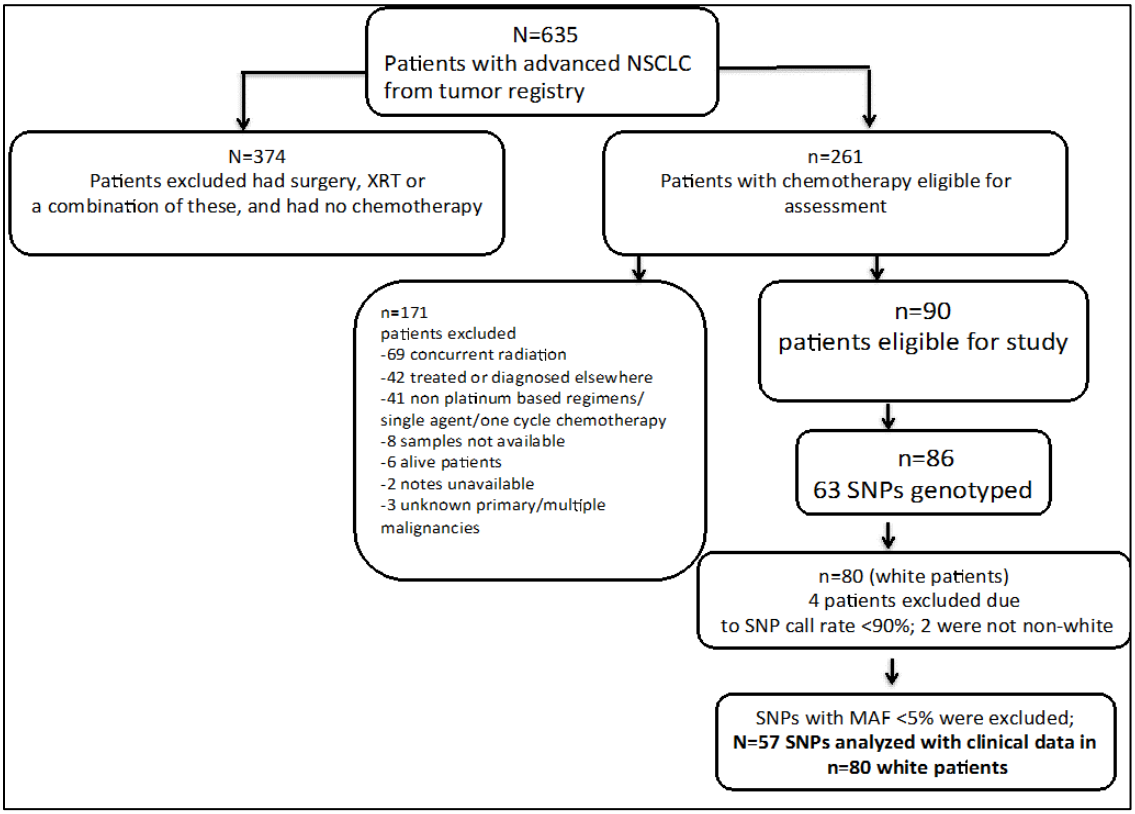

Figure 4.3 Patient selection and Study design.
NSCLC: Non-Small-Cell lung carcinoma, MAF: Minor allele frequency, XRT: Radiotherapy

4.2.2 Treatment schedules, dose & toxicity assessments

Of the 90 patients identified, 87 received carboplatin, two received cisplatin, and two received cisplatin and carboplatin as first-line chemotherapy. Patients were evaluated every 2–3 cycles using the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1. We assessed the following hematologic toxicities: anemia, neutropenia, febrile neutropenia and thrombo-cytopenia. Nonhematologic toxicities assessed included nausea, vomiting, diarrhea, mucositis, sensory and motor neuropathy, renal dysfunction (increase in creat-inine), and liver dysfunction (abnormal liver function tests). Grading of toxicities was performed using the National Cancer Institute Common Toxicity Criteria Version 4.0.Clinical evaluation and blood tests were performed prior to the first cycle of chemotherapy and before each subsequent cycle. Baseline CT scans were obtained before the start of treatment, and repeated every 2–3cycles to evaluate treatment response. Patients had open access to the oncology clinic for reporting any side effects or concerns during treatment.

4.2.3 SNP analysis:

4.2.4 DNA isolation

Genomic DNA was isolated from formalin-fixed paraffin-embedded specimens (n=86 NSCLC patients) using Qiagen DNA isolation from formalin-fixed paraffin-embedded tissues kit (Qiagen, Valencia, CA, USA) as per manufacturer's instructions.

4.2.5 Genotyping

Genes of importance to platinating agents and paclitaxel as well as potentially significant SNPs within them were selected from PharmGKB [19] and literature search from PubMed. Overall, 63 SNPs within 29 genes in 86 subjects with detailed clinical information were genotyped using the Sequenome platform at the University of Minnesota Genomics Center (MN, USA). Only include SNPs with MAF>0.1(**Figure: 4.4, Table 4.1**).

Six samples were removed from further statistical analyses owing to low call rates (<90%). There was no significant difference in age, performance status and survival between patients excluded versus included in study ($p>0.05$). Quality control of the SNP data resulted in one SNP being removed with a call rate <90%, with no SNPs removed owing to deviations from Hardy–Weinberg equilibrium.

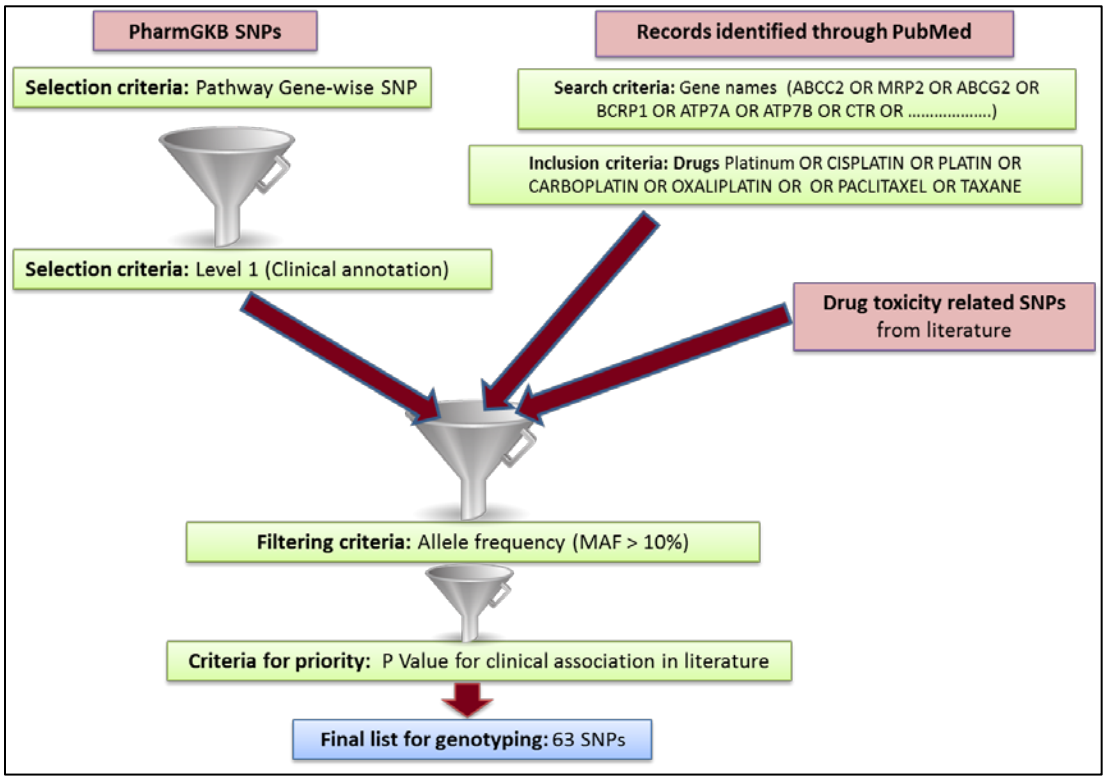

Figure 4.4 SNP selection process.

Table 4.1 Summary of SNPs genotyped in the NSCLC patients.

SNP	Chr	Position	Gene	Major Allele	Minor Allele	MAF	Call rate	HWE
rs1695	11	67352689	GSTP1/missense	A	G	0.336	0.95	0.8
rs7483	1	110279701	GSTM3	G	A	0.342	1	0.802
rs11615	19	45923653	ERCC1/cds-synon	T	C	0.386	0.9875	1
rs13181	19	45854919	KLC3/nearGene-3	T	G	0.431	1	0.821
rs25487	19	44055726	XRCC1/missense	G	A	0.27	0.95	0.151
rs25489	19	44056412	XRCC1/missense	G	A	0.05	1	0.168
rs35592	16	16141823	ABCC1/intron	T	C	0.201	0.9625	0.722
rs119774	16	16086833	ABCC1/intron	G	A	0.069	1	1
rs246240	16	16119024	ABCC1/intron	A	G	0.131	1	0.614
rs316019	6	160670282	SLC22A2/missense	G	T	0.125	1	0.094
rs447978	3	120491086	GTF2E1/intron	A	G	0.312	1	0.117
rs520227	1	190569312		C	G	0.307	0.8875	0.001
rs744154	16	14015081	ERCC4/intron	C	G	0.312	1	1
rs851974	6	151971687	ESR (7 kb 5')	T	C	0.436	1	0.357
rs1042522	17	7579472	TP53/nearGene-5,TP53/missense	G	C	0.291	0.9875	0.584
rs1045385	6	10398116	TFAP2A/UTR-3	A	NA	NA	0.9875	NA
rs1045642	7	87138645	ABCB1	T	C	0.456	1	0.366
rs1047768	13	103504517	ERCC5/cds-synon	C	T	0.342	0.9875	0.624
rs1058930	10	96818119	CYP2C8/missense	C	G	0.069	1	0.308
rs1128503	7	87179601	ABCB1	C	T	0.456	1	0.652
rs1138272	11	67353579	GSTP1/missense	C	T	0.094	1	0.515
rs1347851	12	90566978		A	G	0.244	1	0.542
rs1625895	17	7578115	TP53/intron	G	A	0.144	1	0.193
rs1799735	1	110280254	GSTM3	-	TCC		1	NA
rs1799793	19	45867259	ERCC2/missense	G	A	0.383	0.9625	0.81
rs1800566	16	69745145	NQO1/missense	C	T	0.222	0.9875	0.512
rs1801244	13	52544805	ATP7B/missense	C	G	0.481	0.975	0.82
rs1801247	13	52520471	ATP7B/cds-synon	G	A	0.051	0.9875	1
rs1944118	11	111352032	BTG4/intron	G	A	0.35	1	0.466
rs2031920	10	135339845	CYP2E1/nearGene-5	C	T	0.013	0.95	1
rs2032582*	7	87160618	ABCB1				0.9875	NA
rs2228001	3	14187449	XPC/missense	A	C	0.344	1	0.806
rs2229109	7	87179809	ABCB1	G	A	0.038	1	1
rs2230671**	16	16228242	ABCC1/cds-synon	A	G	NA	0.9875	NA
rs2231142	4	89052323	ABCG2/missense	C	A	0.081	1	0.411

rs2235015	7	87199564	ABCB1	G	T	0.131	1	0.614
rs2238476	16	16213872	ABCC1/intron	C	T	0.062	1	0.259
rs2273697	10	101563815	ABCC2/missense	G	A	0.2	0.9375	1
rs2798389	14	83051150		A	G	0.262	1	0.775
rs3212948	19	45924362	ERCC1/intron	C	G	0.369	1	0.811
rs3212986	19	45912736	CD3EAP/missense,ERCC1/UTR-3	G	T	0.269	0.975	1
rs3740066	10	101604207	ABCC2/cds-synon	G	A	0.308	0.975	1
rs3957357	6	52668687	GSTA1/nearGene-5	G	A	0.394	1	0.35
rs4128317	2	29659854	ALK/intron	A	C	0.419	1	0.818
rs4148396	10	101591944	ABCC2/intron	C	T	0.342	0.95	0.8
rs6863960	5	114995781		G	A	0.469	0.9375	1
rs7255865	19	21804968		A	G	0.412	1	1
rs8187710	10	101611294	ABCC2/missense	G	A	0.056	0.9875	1
rs9282564	7	87229440	ABCB1	A	G	0.088	0.975	0.463
rs9312960	5	204082	CCDC127/5'UTR	C	A	0.294	1	0.282
rs10158985	1	226050609	TMEM63A/intron	C	A	0.247	0.9625	1
rs11572080	10	96827030	CYP2C8/missense	G	A	0.106	1	0.589
rs12188653	5	101383803		G	C	0.257	0.95	0.005
rs13120400	4	89033527	ABCG2/intron	T	C	0.288	1	0.272
rs17098912	14	100104329		G	A	0.206	1	1
rs17222723	10	101595996	ABCC2/missense	T	A	0.051	0.9875	1
rs17718902	11	17784707	KCNC1/intron	A	G	0.388	1	0.642
rs17731538	4	89055379	ABCG2/intron	G	A	0.2	1	0.725
rs28364006	16	16228249	ABCC1/missense	A	G	0.012	1	0.006
rs28730837	17	56355397	MPO/missense	C	T	0.025	1	1
rs45511401	16	16173232	ABCC1/missense	G	T	0.014	1	1
rs72552784	7	87145914	ABCB1	C	NA	NA	1	NA

(MAF= minor allele frequency; HWE= Hardy Weinberg equilibrium)

4.2.6 Statistical methods

All analyses were restricted to subjects with self-reported race of white or 'unknown'. Association of each clinical feature with clinical outcomes of PFS and overall survival (OS) was assessed using Cox-proportion hazards models. Associations of each SNP genotype with clinical outcomes were examined using Cox-proportion hazards models, where the SNP genotyped was coded under a dominant, codominant or additive genetic model. Models were fit with and without adjustment for clinical features associated with clinical outcomes. For analysis of PFS, we included covariates of

stage and performance status. For OS, we included performance status as a covariate. For analyses with clinical features, stage was treated as a categorical variable and performance status was treated as a continuous variable. Age at diagnosis was categorized into four levels based on the 25, 50 and 75% percentiles of the distribution. All statistical analyses were completed using R (version 2.15.3). Adjustment for multiple testing was carried out using Bonferroni correction.

4.3 Results

4.3.1 Demographic features and clinical characteristics

Demographic and clinical features of the 90 evaluable patients at diagnosis are shown in **Table** 4.2. The median age was 66 years (range: 45–81). All the patients were males, a reflection of the patient population at a VA Medical Center. The majority (93%) of patients was white; 81% of patients had stage IV disease. Adeno-carcinoma was the most common histological subtype (42% of patients) of NSCLC, followed by squamous cell (20%) and large cell (16%) carcinoma. The median performance status (ECOG scale) was 1 (range: 0–2).

Table 4.2 Patient demographics and clinical characteristics (n = 90).

	n(%)	p-value (PFS)	p-value (OS)
Age at diagnosis (years)			
Median	66	0.95	0.25
Range	45-81		
Race			
White	84 (93.3)		
African–American	1 (1.1)		
American Indian/Alaska Native	2 (2.2)		
Unknown	3 (3.3)		
Stage			
IIIB	17 (19)	0.02	0.48
IV	73 (81)		
Histology			
Adenocarcinoma	40 (44.4)		
Adenocarcinoma, mucinous	2 (2.2)		
Squamous cell	18 (20)		
Large cell	12 (13.3)		
Large cell neuroendocrine	2 (2.2)		
Non-small-cell carcinoma, NOS	16 (17.8)		
Brain metastasis	12 (13.3)		
Performance status (ECOG)			
Median	1	0.03	0.02
Range	0-2		

ECOG: Eastern Cooperatve Oncology Group; NOS: Not otherwise specified; OS: Overall survival; PFS: Progression-free survival.

4.3.2 Treatment, response and outcomes

Chemotherapy regimens administered, including the number of cycles and average dose intensities of each agent, are shown in **Table 4.3**. The overall response rate was 19%, with a median duration of response of 182days, median PFS of 148 days and median OS of 288 days. No patient achieved a complete response.

Hematological and non-hematological toxicities selected for further analyses are shown in **Table** 4.4. No patients developed treatment-related abnormalities in liver function tests, or experienced CTCAE grade5 (fatal) toxicity.

Table 4.3 Chemotherapy regimens, dose intensity, response and survival.

Regimen	Number of patients	Number of cycles, mean (range)	Dose intensity (mg/m2/week)†, mean ± SE		Response rate, n (%)	Median duration of response (days)	Median PFS (days)	Median OS (days)
			Platinum‡	Second drug				
C + T	77	3.3 (1–6)	1.8 ± 0.3	60.0 ± 10.0	14 (18)	163	148	292
C + G	9	3.2 (1–5)	1.4 ± 0.4	455.1 ± 169.1	3 (33)	185	211	465
C + E	2	3 (1–3)	1.9 ± 0.5	84.9 ± 19.0	0 (0)	0	NE	NE
Cis + E	2	1.5 (1–2)	21.8 ± 4.2	75.0 ± 0.0	0 (0)	0	NE	NE
Total	90	3.2 (1–6)	–	–	17 (19)	182	148	288

None of the patients had a complete response.
The median PFS and OS were calculated from the Kaplan–Meier estimates.
†The target dose intensities for each agent were: C AUC 2/week; Cis, 27 mg/m2/week; T, 67 mg/m2/week; G, 667 mg/m2/week and E, 100 mg/m2/week (with C) and 80 mg/m2/week (with Cis).
‡Dose of C is expressed as AUC.
AUC: Area under the concentration–time curve; C: carboplatin; Cis: Cisplatin; E: Etoposide; G: Gemcitabine; NE: Not evaluable; OS: Overall survival; PFS: Progression-free survival; SE: Standard error; T: paclitaxel.

Table 4.4 Hematological and non-hematological toxicities.

Toxicity and grade†	Chemotherapy regimen			
	C + T, n (%)	C + G, n (%)	C + E, n (%)	Cis + E, n (%)
Neutropenia				
1–2	10 (12)	5 (56)	0 (0)	0 (0)
3–4	19 (25)	2 (22)	1 (50)	0 (0)
Febrile neutropenia				
1–2	0 (0)	0 (0)	0 (0)	0 (0)
3–4	3 (4)	0 (0)	0 (0)	1 (50)
Anemia				
1–2	72 (94)	7 (78)	2 (100)	0 (0)
3–4	2 (3)	2 (22)	0 (0)	1 (50)
Thrombocytopenia				
1–2	18 (23)	4 (44)	0 (0)	1 (50)
3–4	3 (4)	2 (22)	0 (0)	1 (50)
Nausea				
1–2	28 (36)	3 (33)	0 (0)	2 (100)
3–4	5 (7)	0 (0)	0 (0)	0 (0)
Vomiting				
1–2	4 (5)	1 (11)	0 (0)	0 (0)
3–4	4 (5)	0 (0)	0 (0)	0 (0)
Diarrhea				
1–2	15 (20)	2 (22)	0 (0)	0 (0)
3–4	7 (10)	0 (0)	1 (50)	1 (50)
Mucositis				
1–2	1 (1)	1 (11)	0 (0)	0 (0)
3–4	0 (0)	0 (0)	0 (0)	0 (0)
Sensory neuropathy				
1–2	24 (31)	3 (33)	0 (0)	0 (0)
3–4	5 (7)	0 (0)	0 (0)	0 (0)
Motor neuropathy				
1–2	0 (0)	0 (0)	0 (0)	0 (0)
3–4	2 (3)	0 (0)	0 (0)	0 (0)
Increased creatinine				
1–2	2 (3)	1 (11)	0 (0)	0 (0)

†Toxicities were graded according to Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0. None of the patients developed abnormal liver function tests or experienced grade 5 toxicity. The number of patients who received each chemotherapy regimen was: C + T = 77, C + G = 9, C + E = 2 and Cis + E = 2. C: carboplatin; Cis: Cisplatin; E: Etoposide; G: Gemcitabine; T: paclitaxel.

4.3.3 Pharmacogenetics

We first examined the association of known clinical prognostic factors (covariates) with clinical outcomes. Stage (p=0.02) and performance status (p=0.03) were predictive of PFS, and performance status (p=0.02) was associated with OS. Therefore, we adjusted for these covariates when analyzing the association between SNPs and outcomes. In the analysis of toxicity, we adjusted for the total number of cycles received, since the cumulative dose of chemotherapeutic agents received would be expected to increase the risk of toxicities such as peripheral neuropathy. For genetic association testing, 86 patients had both clinical and genotype data. Of these 86 patients we included patients with white ancestry (n=80) for further analysis. Of 58 SNPs with a minimum allele frequency of >0.05, one SNP was excluded due to call rate of <0.90. Although number of chemotherapy cycles were associated with OS, we decided not to adjust for them as genetic factors might be influencing number of cycle and if we adjust for cycles, we might mask some of the effects due to patient genetics (as it might be 'surrogate' for length of survival[depends on time; changes over time]).PFS was associated with SNPs in drug efflux transporters ABCC1 (intronic SNPs: rs246240 and rs2238476) and ABCB1 (coding synonymous SNP, rs1128503); a Quinone reductase NQO1 (missense

SNP, rs1800566); TMEM63A (rs10158985); and other genes such as KCNC1 (rs17718902) and CCDC127 (rs9312960; summarized in **Table 4.5**). SNPs within ABCB1 (rs1045642; p=0.04), ABCC1 (rs2238476; p=0.03), ABCG2 (rs17731538; p=0.04) and TMEM63A (rs10158985; p=0.03) were also associated with OS.

We also evaluated association of SNPs with major hematological and non-hematological toxicities. Toxicities were not associated with the initial performances status (p>0.05) but were associated with age and number of treatment cycles. After adjusting for age and number of treatment cycles, we found associations of ABCB1SNP with thrombocytopenia (rs2235015; p=0.04), and ABCG2

(rs2231142; p=0.045) and ATP7B (rs1801244; p=0.027) SNPs with nausea (**Table 4.6**), and ABCG2SNP (rs13120400, p=0.027) with sensory neuropathy, respectively. Interestingly, SNPs in the DNA repair pathway genes ERCC4 (rs744154; p=0.04) and XPC (rs2228001; p=0.045) demonstrated associations with neutropenia and sensory neuropathy, respectively (**Table 4.6**). Additionally, we observed SNPs in TP53 (rs165895; p=0.02), CYP2C8 (rs11572080; p=0.017) and CCDC127 (rs9312960; p=0.019) to be associated with diarrhea. rs447978, an intronic SNP in transcription factor GTF2E1, was associated with both neutropenia (p=0.024) and nausea (p=0.027). After adjustment for multiple testing (Bonferroni) the above indicated associations were not significant and the results need to be validated in a larger study.

Table 4.5 Association of SNPs with progression-free survival after adjusting for covariates (performance status and stage).

SNP	Gene	Drug	Chr	Major Allele	Minor Allele	MAF	P value PFS Additive Model *(Dominant Model)*	Hazard Ratio PFS Additive Model *(Dominant Model)*	95% CI, additive model (dominant model)
rs1800566 (missense)	NQO	platinating agents	16	C	T	0.222	0.02 (*0.037*)	1.615 *(1.689)*	0.089 to 0.870 (0.040 to 1.009)
rs10158985 (intron)	TMEM63A[#]	platinating agents	1	C	A	0.247	0.032 (*0.025*)	0.655 *(0.592)*	-0.821 to -0.025
rs246240 (intron)	ABCC1	Taxane	16	A	G	0.131	0.037 (0.072)	0.640 *(0.617)*	0.409 to 1.001
rs2238476 (intron)	ABCC1	Taxane	16	C	T	0.062	0.045 (*0.037*)	1.972 *(2.32)*	-0.894 to 0.001
rs1128503 (synonymous)	ABCB1	Taxane	7	C	T	0.456	0.066 (0.019)	0.730 *(0.541)*	-0.652 to 0.022
rs17718902 (intron)	KCNC1[#]	platinating agents	11	A	G	0.388	0.069 (0.035)	1.353 *(1.717)*	-0.017 to 0.623
rs9312960	CCDC1278[#]	platinating agents	5				0.059 (0.042)	1.378 *(1.620)*	-0.003 to 0.645

Data are presented as: values from additive model, three group analysis (values from dominant model, variant carrier vs noncarrier).
MAF: Minimum allele frequency.

Table 4.6 Association of SNPs with toxicity after adjusting for covariates (age as categorical variable and total number of cycles).

Toxicity	Gene	SNP	P value, log Additive Model *(Dominant Model)*	Odds Ratio Additive Model *(Dominant Model)*	95% CI, log-additive model (dominant model)
Nausea	ABCG2	rs2231142_CA (missense)	0.045*(0.045)*	3.938 (4.052)	0.033 to 2.708 (0.031 to 2.768)
	GTF2E1	rs447978_AG (intron)	0.024 *(0.004)*	0.409 (0.223)	-1.672 to -0.117 (-2.520 to -0.488)
	ATP7B	rs1801244_CG (missense)	*0.078 (0.027)*	1.977 *(4.626)*	-0.073 to 1.384(0.178 to 2.885)
Neutropenia	GTF2E1#	rs447978 (intron)	0.027 *(0.039)*	0.444 *(0.345)*	-1.531 to -0.093(-2.01 to -0.053)
	TMEM63A	rs10158985	0.031 (0.09)	2.557 *(2.307)*	0.035 to 1.52(0.308 to 2.307)
	ERCC4	rs744154 (intron)	0.04 *(0.01)*	2.176 (3.697)	0.087 to 1.79(-0.158 to 1.83)
Sensory Neuropathy	#	rs1347851_AG	0.016 (0.023)	0.235 *(0.225)*	-2.625 to -0.27(-2.773 to -0.208)
	ABCG2	rs13120400 (Intron)	0.027 (0.055)	0.271 (0.295)	-2.467 to -0.146(-2.465 to 0.024)
	KLC3#	rs13181_TG	*0.073 (0.021)*	2.27 *(5.893)*	-0.076 to 1.715 (0.273 to 3.274)
	XPC	rs2228001_AC (missense)	*0.096 (0.045)*	0.456 *(0.296)*	-1.71 to 0.14(-2.406 to -0.03)
Diarrhea	CCDC127	rs9312960_CA (5'UTR)	0.019 (0.09)	2.429 (2.434)	0.149 to 1.626 (-0.138 to 1.918)
	CYP2C8	rs11572080_GA (missense)	0.017 (0.017)	4.396 (4.396)	0.26 to 2.702 (0.26 to 2.702)
	TP53	rs1625895_GA (intron)	0.02 (0.023)	3.004 (3.633)	0.174 to 2.026 (0.179 to 2.401)
Thrombocytopenia	ABCB1	rs2235015_GT	*0.074 (0.04)*	2.605 (3.467)	-0.093 to 2.004 (0.058 to 2.428)
	#	rs17098912_GA	*0.072 (0.027)*	2.212 (3.115)	-0.071 to 1.659 (0.131 to 2.142)

Data are presented as: values from additive model, three group analysis (values from dominant model, variant carrier vs non-carrier).
†SNPs not in a gene, these were selected from genome-wide association study.

4.4 Discussion

This comprehensive analysis identified pharmacogenomics variants of clinical significance within key genes (n=29) in the platinating agents pathway and taxane pathway in NSCLC patients treated with a combination of carboplatin and paclitaxel. We found significant associations of drug transporter (ABCC1and ABCB1) SNPs with PFS. Although we did not observe significant association of ABCB1rs1045642 with outcome/toxicity, ABCB1SNPs rs1128503 (occurring in partial linkage disequilibrium with rs1045642) and rs2235015 were associated with PFS and thrombocytopenia, respectively. The functional consequence of the three most commonly studied SNPs (rs1128503, rs1045642 and rs2032582 also occurring in linkage disequilibrium) in ABCB1is still not completely understood. Associations of these SNPs with mRNA/protein expression have been shown in some studies but not all the studies. rs1045642, a synonymous SNP (3435C>T; Ile1145Ile) has also been indicated to influence protein conformation and substrate specificity [20]. Previous studies have identified association of ABCB1SNPs (especially rs1045642) with survival in osteosarcoma patients treated with cisplatin containing chemotherapy [21]. In esophageal cancer patients treated with platinating agents, presence of the T allele for rs1045642 is associated with significantly longer survival and reduced risk of recurrence [22]. Together, these findings suggest that drug transporter SNPs that might influence outcomes in several malignancies by altering intracellular drug concentrations.NQO1 belongs to the Quinone dehydrogenase family of proteins and has been implicated in metabolizing platinum agents. A missense SNP rs1800566 (NQO1*2: Pro187Ser) is in the active site of the enzyme and has been associated with reduced NQO1 activity. The NQO1 isoform with Ser (NQO1*2) undergoes rapid degradation [23, 24]. A recent study reported association of the NQO1*2SNP with shorter OS in NSCLC patients treated with adjuvant radiation therapy with or without platinating agent based chemotherapy [25]. Our results are in concordance with these previous observations, with presence of NQO1*2associated with poor PFS (**Table 4.5**).Toxicity due to platinating agent and taxane-based chemotherapy remains a major challenge

faced by clinicians and patients. There are no well-established biomarkers that can predict such toxicities in individual patients. In our efforts to identify SNPs predictive of major hematological and non-hematological toxicities, we observed that a missense SNP in CYP2C8, a drug-metabolizing enzyme involved in the metabolism of taxanes, was significantly associated with higher incidence of diarrhea (OR: 4.396; p=0.017). Previous results in breast cancer patients receiving paclitaxel have shown significant association of CYP2C8*3(which denotes two highly linked SNPs rs11572080 and rs10509681) with better response and a trend towards greater risk of peripheral neuropathy [26]. CYP2C8*3 is associated with lower paclitaxel-α hydroxylation activity, and hence carriers of this SNP have lower clearance of paclitaxel, which can contribute to better response but also greater risk of toxicity to normal cells [27, 28] In addition to drug-metabolizing enzymes, several SNPs in drug transporters (ABCG2 and ABCB1) were associated with nausea and thrombocytopenia, respectively. These drug transporters have been implicated in efflux of either platinating agents or taxanes. The ABCG2missense SNP rs2231142 results in a Gln141Lys change; which has been associated with lower expression [29] as well as with reduced drug efflux capacity of ABCG2 [30]. Cell lines that are resistant to cisplatin have been shown to express higher levels of the copper transporters ATP7Aand ATP7B [5]. ATP7A has been implicated in sequestering both carboplatin and cisplatin in vesicles. Since SNPs in these transporters have not been evaluated for potential clinical impact, we performed an exploratory evaluation of SNPs in ATP7Aand ATP7Band found that rs1801244 (valine to leucine change) in ATP7B is associated with nausea. The functional effect of the valine to leucine change resulting from this SNP remains to be determined. Since platinating agents such as cisplatin and carboplatin form platinum–DNA adducts that are repaired by the nucleotide excision repair pathway, interpatient variation in DNA repair mechanism due to presence of SNPs in DNA damage/repair pathway genes may influence treatment outcome. Although none of the SNPs in the DNA repair pathway genes were associated with PFS or OS, significant correlations were observed with toxicities. A 3′-UTR SNP (rs3212986)

and a coding SNP in ERCC1(rs11615 ; Asn118Asn) have been associated with reduced ERCC1 mRNA/protein-expression levels, and lower ERCC1 levels have been associated with better outcomes in NSCLC patients receiving platinating agent based chemotherapy [31, 32]. Nevertheless, while multiple studies have evaluated these two ERCC1SNPs, results have been quite variable and remain inconclusive. A recent meta-analysis of 39 previously published studies on these two ERCC1SNPs in lung cancer patients demonstrated that the rs11615 SNP might be a potential biomarker for risk of developing lung cancer as well as a prognostic marker in NSCLC patients treated with platinating agents [33]. In our dataset we did not observe significant association of these SNPs with toxicity of survival. We did observe a marginally increased risk of neutropenia with ERCC4intronic SNP and reduced risk of sensory neuropathy with XPC missense SNP rs2228001 (C>A; Gln939Lys). Though not extensively studied, rs2228001 has been shown to have a trend towards higher risk of ototoxicity in osteosarcoma patients treated with cisplatin [34].Finally, in addition to candidate genes in drug pathways; we tested selected genes that have been identified by genome-wide association study (GWAS). GWAS analysis in colorectal cancer patients treated with 5-fluorouracil with or without oxaliplatin identified SNPs in KCNC1 (rs17718902), GTF2E1 (rs447978), CCDC127rs9312960) or TMEM63A (rs10158985) to be associated with nausea, vomiting and drug-induced neuropathy [35]. In our study, an intronic SNP (rs447978) in the transcription factor GTF2E1 was associated with reduced risk of nausea and neutropenia and an intronic SNP in TMEM63 (rs10158985) was associated with neutropenia. Furthermore, SNPs in KCL3and CCDC127were associated with increased risk of sensory neuropathy and diarrhea, respectively. Although the functional relevance of these genes/SNPs is not known, future studies on functional characterization as well as validation of these SNPs are needed to confirm the results of the GWAS analysis.

4.5 Conclusion

In conclusion, we identified SNPs within key candidate drug pathway genes that are independently predictive of PFS and/or major hematological and non-hematological toxicities in patients with advanced NSCLC treated with platinating agent based chemotherapy. There is a paucity of predictive markers that can be used to guide clinical decisions for such patients. Our results confirm some previously reported associations, but more importantly identify several new candidates that warrant testing in prospective studies, that could contribute towards the development of personalized medicine. Indeed, a recent Phase II trial demonstrated that response rates are higher when chemotherapy is selected based on SNPs in ERCC1and RR M1in patients with advanced NSCLC [36], suggesting that incorporation of pharmacogenomics biomarkers into clinical decision making has considerable potential for improving therapeutic outcomes.

4.6 Summary

Lung carcinoma is the most common malignancy and the leading cause of cancer deaths worldwide. The genetic makeup of individual patients in addition to other clinical factors can influence the likelihood of benefit from and tolerability of chemotherapy. We evaluated SNPs in candidate genes of relevance to platinating agents and paclitaxel for association with survival and toxicity in non-small-cell lung cancer patients. After adjusting for covariates, SNPs in drug transporters and NQO1 were associated with progression-free survival and SNPs in drug transporters as well as in the in the DNA repair pathway genes ERCC4 and XPC were associated with toxicity. Comprehensive evaluation of the genetic variants in conjunction with known prognostic factors may help optimize therapeutic decisions to maximize benefit and minimize toxicity in non-small-cell lung cancer patients.

CHAPTER 5 SUMMARY

Platinum drugs are currently used as standard-of-care chemotherapy for various cancers including solid tumors like lung cancer, the most common malignancy in the world, and ovarian cancer, the most lethal gynecological malignancy in the United States. Platinating agents including carboplatin form DNA helix-distorting DNA adducts that lead to strand breakage activating DNA repair mechanisms that ultimately resulting in apoptotic death of cancer cells. Taxanes are another widely used class of chemotherapy drug to treat various cancers including ovarian and lung cancers, primarily in combination with platinating agents. paclitaxel, a taxane, is a mitotic inhibitor that stabilizes microtubules and interferes with cell division leading to induction of apoptosis.

However, despite considerable improvements in therapeutic approaches, the overall response rate of carboplatin/paclitaxel based combination chemotherapy is far from desirable. Drug resistance, interpatient variation in response and toxicity are major causes of concern. Furthermore, a large bulk of the cancer patients who do respond eventually undergo relapse after first line based chemotherapy. Inter-patient variability in treatment response is therefore a commonly observed phenomenon in this drug resistance. However, the complete mechanism governing this spectrum of sensitivity to chemotherapeutic agents still remains largely unclear.

Genetic factors like differential expression and/or activity of genes involved in drug metabolism pathway and the presence of single nucleotide polymorphisms may have an impact on treatment outcome and toxicity in cancer patients being treated with Platinum/Paclitaxel combination. Genetic variation in these key pharmacokinetic and pharmacodynamics pathway genes may therefore contribute to interpatient variation in response and toxicity.

Our approach was to use cell line models derived from epithelial ovarian cancer (EOC) patients to identify genetic polymorphisms and differential gene expression associated with chemo-sensitivity and response to carboplatin and paclitaxel single agent and combination treatments followed by the evaluation of these predictive pharmacogenomics markers in epithelial ovarian cancer and NSCLC patients undergoing Carbplatin/Paclitaxel combination chemotherapy. For the purpose of cell-

based modeling of ovarian cancer, we generated immortalized Lymphoblastoid cell lines (LCLs) from human lymphocytes from Epithelial ovarian cancer (EOC) patients through the infection of Epstein-Barr virus (EBV). Then, we performed a comprehensive profiling in vitro chemo-sensitivity phenotypes in our panel of ~100 Epstein-Barr virus (EBV) transformed EOC LCLs following treatment with carboplatin, paclitaxel as single agent and in combination. We demonstrated extensive inter-individual variation in drug response as was evident from the drug IC_{50} and area under survival curve (AUC) values. Fold changes between the lowest (most-sensitive) and highest (most-resistant) IC_{50} and AUC values were 8.34 & 3.84, respectively for carboplatin, and 7.72 & 5.65, respectively for paclitaxel**.** Our drug cytotoxicity values corroborated with the caspase activity assay results demonstrating that drug cytotoxicity indeed reflected alterations in cellular apoptosis and programmed cell death. Furthermore, using Chou-Talalay's Combination index-Isobologram Theorem we also observed, based on Combination index values, wide inter-individual variations in effects of carboplatin and paclitaxel when used in combination treatment. The wide range of dose reduction index values indicated variability in impact of the dose of one drug on another in combination treatment based on the cell line used for the study.

To understand the pharmacogenomics of the inter-individual variation in chemo-sensitivity parameters, we performed a pathway based identification of polymorphisms within the PK/PD pathway of carboplatin and paclitaxel as well as analysis of gene expression of these pathway genes in the EOC LCLs using TaqMan Low Density Arrays (TLDA), followed by genotype-phenotype association analysis.

Our analysis revealed significant association between drug chemosensitivity measures and genetic variations in several key candidate genes involved in carboplatin and paclitaxel pathway.

Genetic variations in drug transporter genes ABCG2 and ABCC2, several mutations in the DNA repair gene ERCC6 and XRCC5 as well as mutations in PMS2 and MSH2 and MSH6 were found associated with carboplatin cytotoxicity whereas. Mutations in microtubule associated protein

MAP4, the transporter genes ABCC1 and SLC22A7, as well as TP53 and EGFR were associated with paclitaxel response. Top among the mutations associated with combination treatment were SNPs within the genes ABCC1, MSH2 and MSH6. Remarkably, we found a number of coding variants associated with drug response. These include K1087N (MAP4; paclitaxel), F150F (TP53; paclitaxel) and S667S (ABCC1; combination), R704R (ERCC6; carboplatin), R338R and P428P (MSH6; carboplatin). Our gene expression analysis revealed changes in expression levels of the following pathway genes were associated with drug response phenotypes: ERCC2 (carboplatin), GSTM1 (carboplatin) and NQO1 (carboplatin); FOXC2 (paclitaxel), FOXL1 (paclitaxel) and BIRC5 (paclitaxel). NER genes have been shown to play significant roles in the identification and repair of platinum-DNA adducts. Earlier studies have shown upregulation of expression of the NER pathway genes ERCC1 and XPD was significantly associated with higher resistance in platinum-based chemotherapy in advanced EOC. Our results thus corroborate with this earlier finding and identifies the NER genes associated with platinum drug resistance.

Subsequently, we performed a comprehensive genome-wide association scans (GWAS) of germline genotype of these patient-derived LCLs to discover predictive pharmacogenomics markers of treatment response within the human genome. At $p < 10^{-4}$, we found genes related to malignant solid tumor and epithelial cancers that were significantly associated with our drug chemosensitivity measures. Notably, the top canonical pathways represented by these genes included Epithelial Adherens Junction Signaling, Sertoli Cell Junction Signaling and Endometrial Cancer Signaling, which seems reasonable as the cells were derived from ovarian cancer patients. We also found mutations in several relevant genes associated with drug response including the tumor suppressor genes CTNNA2, CDH4 and LRP1B; FRAS1, a gene supposedly involved in tumorigenesis and metastasis; and the DNA repair gene BRE.

Subsequently, to understand the impact of genetic variation within the pharmacokinetic/pharmacodynamics pathways of platinating agents and taxanes in NSCLC, we

performed a genotype-phenotype correlation analysis on in advanced NSCLC patients treated primarily with carboplatin/paclitaxel-based combination chemotherapy. Our results showed a number of pathway SNPs associated with treatment outcomes and toxicity including progression-free survival (PFS) and multiple adverse effects, following adjusting for clinical prognostic factors in multivariate models. Our results showed SNPs in key pathway genes were associated with treatment outcome (PFS) including ABCC1, ABCB1 (rs1045642/ Ile1145Ile, rs1128503, rs2235015), NQO1 (NQO1*2: Pro187Ser). Whereas, SNPs in the following genes were also associated with hematological and/or non-hematological toxicities: CYP2C8 (diarrhea); ABCG2 and ABCB1 (thrombocytopenia); (ATP7B rs1801244; nausea); ERCC4 (neutropenia); XPC missense SNP rs2228001 (C>A; Gln939Lys). Among other SNPs associated with toxicities included rs447978 (GTF2E1; nausea and neutropenia, rs10158985 (TMEM63; neutropenia), as wells as SNPs in KCL3 (neuropathy) and CCDC127 (diarrhea). The nonsynonymous coding SNP in NQO1, rs1800566 (NQO1*2: Pro187Ser), is in the active site of the NQO1 enzyme and has been associated with reduced NQO1 activity since the NQO1 isoform with Ser (NQO1*2) undergoes rapid degradation.

Most remarkably, the expression of NQO1 was also shown to be associated with our *in vitro* profiling of carboplatin chemosensitivity in EBV-transformed ovarian cancer LCLs. Similarly, SNPs in a number of other pathway genes that were found relevant with regard to the treatment response and/or toxicities resulting from treatment with platinating agents/ taxane-containing chemotherapy regimens in NSCLC patients were also shown associated with *in vitro* chemosensitivity in our EOC LCL panel-based model system of drug resistance. For example, the ABCC1 intronic SNPs rs246240 and rs2238476 were associated with PFS in NSCLC patients after adjusting for covariates, while ABCC1 SNPs were also found significantly correlated with *in vitro* paclitaxel and combination cytotoxicity as well as caspase activity following combination treatment. The ABCG2 missense SNP rs2231142 was associated with nausea, and the intronic SNP

rs13120400 with Sensory neuropathy in NSCLC; ABCG2 SNPs were also associated with in vitro carboplatin cytotoxicity. In addition, SNPs in CYP2C8 and TP53 were associated with diarrhea in NSCLC patients as well as response to paclitaxel *in vitro.* Also, ERCC2 SNPs were associated with caspase 3/7 activity following carboplatin treatment *in vitro*, while the ERCC2/KLC3 missense SNP rs13181 that was found significantly associated with sensory neuropathy in NSCLC patients after adjusting for covariates.

Thus, using immortalized LCLs directly derived from patient subjects we could successfully develop *in vitro* drug response models that could be directly correlated with patient clinical responses. Furthermore, we have effectively demonstrated by using candidate-gene based and genome-wide approaches in these immortalized LCLs from patient samples we could identify key pharmacogenomics changes associated with drug response, treatment outcome and toxicity. Finally, we identified genetic variation within the pharmacokinetic/pharmacodynamics pathways of platinating agents and taxanes in real world clinical settings in advanced NSCLC patients undergoing carboplatin-based chemotherapy.

However, although we found a number of genetic variations significantly associated with treatment outcome and toxicities, the functional relevance of most of these SNPs vis-à-vis activity of target genes is still largely unknown. Therefore, for a comprehensive understanding of the role of these variations, we propose future studies focusing on the characterization of the significant SNPs using functional genetic/genomic approaches.

Therefore, results from our study provide an actionable panel of reliable genetic biomarkers of carboplatin and paclitaxel single-agent and combination treatment response as clinically applicable signatures of drug response and toxicity which has the potential to be used in precision medicine approaches following further validation to develop pharmacogenomics-guided treatment approaches to achieve maximum efficacy and minimum toxicity.

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Chapter 1

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Chapter 4

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